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STUDIES ON A CRYPTOBIID HEMOFLAGELLATE FROM MARINE FISHES OF NORTHERN NEWENGLAND

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BY

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SECTION I

INTRODUCTION

Extending over great ecological ranges and numerous parasitic habitats, many organisms of the family Cryptobiidae have been found since 1846 when Leidy described Cryptobia helicis n. g., n. sp., from the seminal receptacle of a land snail. Parasites of this genus have also been recorded from the blood of freshwater fish (Laveran and Mesnil, 1901) and salamanders (Rankin, 1937), the digestive tract of some marine fish (Leger, 1905), and as ectoparasites on goldfish (Swezy, 1919). Two species of Cryptobia have also been reported as free living in brackish water (Ruinen, 1938).

The parasite used in this study was first found by Dr. Wilbur Bullock on February 9, 1952, in the blood of a 4½ inch winter flounder caught through the ice at Great Bay, Greenland, N. H. He described the organism briefly as being about 18 micra long, twice the length of the oval red blood cells of the fish (Bullock, 1952a and 1952b). In stained preparations the parasites were usually C-shaped, with a prominent darkly staining kinetoplast on the inner curve. A slightly larger, paler, more granular trophonucleus is located at about the same level on the outer curve. Both flagella arise at the anterior end. The anterior one is

completely free, while the posterior one proceeds down an undulating membrane, extending out the posterior end. Bullock proposed that the name of the species, when adequately described, be Cryptobia newingtoniensis. Since no adequate description has been published, this name has only the status of a nominum nudum.

The first objective of this study is to identify the parasite taxonomically. Before any proposed name assumes a permanent taxonomic status, however, the organism must be adequately described and compared with known species from freshwater and marine hosts. Since Bullock's first report of the organism in the winter flounder, (Pseudopleuronectes americanus), he has also found the parasite in the smooth flounder, (Liopsetta putnami), in two species of Fundulus, and in the grubby (Myoxocephalus aeneus). It therefore becomes necessary to determine the host range of the flagellate as such data is of value in the taxonomic identification of the parasite.

The second objective of the study is to investigate the natural history of the parasite. Early reports of the life cycles of Cryptobia (Trypanoplasma) in freshwater fish point out the role of leeches as transmitting agents. Brumpt (1906b), Keysselitz (1906), Robertson (1911), and others have investigated the development of the flagellates in these animals. A logical approach to the life history of this new species of flagellate was to examine the leeches found in Great Bay. This aspect of the study was difficult

to accomplish because the leeches were scarce. In no instances were the animals found on fish obtained by seining, or by the laborious task of examining bottom samples from the bay. The most fruitful method of collecting the leeches was that of catching flounders with hook and line. Occasionally the leeches were found attached to the fish. Even though a few specimens were finally obtained, they refused to feed on flounders for periods of several months. Under these conditions it was impossible to investigate the multiplication of the flagellates within the leech; therefore, this area remains to be pursued. Knowledge is lacking concerning other possible intermediate hosts and the longevity of infection in fishes. Adequate descriptions of multiplication of the flagellates in the vertebrate hosts are meager in the published literature. Investigation of this problem is undertaken in this study, also.

A third objective of the study is to determine the pathological effects of the flagellate on the fish. Laveran (1904) first showed the ability of Trypanoplasma borreli to kill fish. More recently Wales and Wolf (1955) reported high mortality in yearling king salmon due to Cryptobia infections. Because natural infections proved difficult to perpetuate in the laboratory, I resorted to artificial infections as a means of determining the virulence of the flagellates.

The study of this species of Cryptobia takes on added interest because the only other published report of

Cryptobia (Trypanoplasma) in marine fish is that of Mackerras and Mackerras (1925). They described Trypanoplasma parmae from an Australian marine teleost, Parma microlepsis. This dissertation then, describes the first species of Cryptobia found in marine fish in this hemisphere and the second species in the world. In fact few species of this flagellate from freshwater vertebrates have ever been reported from North America.

Because this flagellate is apparently uncommon, the fourth objective of this investigation is to determine its geographic distribution. As Bullock was unable to find the organism in fish from the Cape Cod area of Massachusetts, it became conceivable that the parasite was restricted to the Great Bay region of New Hampshire. Therefore, fish were examined from other areas of brackish and normal salinities, ranging from Cape Cod, Mass., to Mt. Desert, Maine.

The incidence of Cryptobia infection in flounders of Great Bay, New Hampshire was studied, as well as its relationship to size and age of the vertebrate hosts. The possibility of seasonal parasitism also required investigation. The problem of host resistance to the parasite was largely untouched.

Generally then, the study reported in this dissertation was intended to explore the biology of this new and interesting species of blood parasite and to serve as a foundation for further investigation of the unanswered questions raised by this research.

SECTION II

REVIEW OF LITERATURE

The biflagellate nature of the organism under study is immediately apparent. A properly stained preparation shows that one flagellum extends out freely from a point close to the kinetoplast, whereas the second flagellum prolongs itself along the edge of an undulating membrane to become free at the opposite end of the organism. This characteristic alone distinguished the animal taxonomically from the genus Trypanosoma which is equipped with only a single flagellum bordering on an undulating membrane.

Taxonomic confusion is obvious immediately upon reviewing the early literature of biflagellate organisms, for there are apparently interchangeable names for the same genus.

The first report in the literature that concerns itself with protozoan parasites of the blood of fish is that of Valentin (1841). In his record of an "entozoan" in the blood of trout he described amoeba-like organisms. While his sketches lack detail, the organism depicted is suggestive of a flagellate and is the first reference to any trypanosome or possibly to the genus now known as Cryptobia.

The genus Cryptobia was established in September, 1846, when Dr. Joseph Leidy, the Philadelphia physician and

natural scientist, described a new genus and species of "Entozoa", fundamentally fusiform with a flagellum at each end of the organism. His paper, published in the Proceedings of the Academy of Natural Sciences of Philadelphia, reads as follows:

> Cryptobia. Animal minute; form exceedingly proteoid; internal organization cellular or granular.
> C. helicis. Colorless; form, ordinarily elongate, ellipsoid, fusiform, or ovate; caudated, caudae opposite, one longer than the other. Internal granular structure consisting of two large cells and numerous minute granules. Total length from the one hundred and twenty-sixth to the one-hundredth of a line. Habitat, the vessie copulatrice or spermatheca of Helix albolabris (Polygyra albolabris (Say)), Helix tridentata (Polygyra tridentata (Say)) and Helix alternata (Pyramidula alternata (Say)).

Leidy also indicated the intense ability of the organism to change its shape and presented illustrations of the organism, whose name Cryptobia was derived from the Greek "hidden" and "to live."

It appears that the generic name of Cryptobia was destined for disorder, however, for a year later in August, 1847, in the same periodical, "Dr. Leidy requested permission, which was given, to change the name of the new genus of Entozoa, described by him in Vol. 3, No. 5, of the Proceedings, from that of Cryptobia to Cryptoicus, the former name having been preoccupied." (Leidy, 1847a). Actually the former name to which he referred was Cryptobium Mannerheim, 1831; and Cryptobias Dupont, 1834, both of which are beetles (Coleoptera).

Later (1847b) Leidy extended the host range of Cryptoicus to include Helix elevata (Polygyra elevata Say),

Helix thyroidus (Polygyra thyroidus Say) and Bulimus decollatus (Rumina decollata Linnaeus). Leidy also added the following comment, "Sometimes it is collected in bunches adhering by the end of one of the cauda to each other and frequently it may be observed to contract upon either of the long cellules, causing them to project beyond the outline of the animal."

An interesting development occurred in 1850 when Diesing, in his Systema helminthum named the organism Bodo (Cercomonas)helicis Diesing. Apparently Diesing saw no need to erect a new genus, as the members of the family Bodonidae are biflagellate organisms, largely free-living, and without undulating membranes. It is somewhat surprising to note in the Proceedings of the Academy, 1851, that Leidy himself accepted the correction and approved of Diesing's action. Furthermore his acquiescence was reiterated in 1856. Even though the name Cryptobia was changed to Cryptoicus and again to Bodo, these alterations were unjustified because there actually was no conflict with the original designation of Cryptobia.

An organism was described in 1860 by Keferstein and Ehlers from the bursa copulatrix of Helix pomatis as an "infusorian." From the account given, the organism could easily be placed in the genus Cryptobia of Leidy, 1846. However, apparently no attempt was made to name the parasite specifically.

In 1888 Chalachnikow described varieties of trypan-

osomes with two flagella from the blood of fish. His examinations were largely of fresh blood or incompletely stained preparations, and consequently structural detail was difficult to observe. Kunster (1898) also described a trypanosome from Cobaye which had a flagellum at each end.

The taxonomy of biflagellate blood protozoa was further confused by Laveran and Mesnil (1901) when they erected the new genus Trypanoplasma for parasites found in the blood of freshwater fish.

Nous avons créé, en 1901, le genre Trypanoplasma pour un flagellé du sang du rotengle, Scardinius erythrophtalmus, qui se distingue nettement des parasites du genre Trypanosoma par l'existence de 2 flagelles, l'un antérieur et l'autre postérieur, nous avons désigné le trypanoplasme du rotengle sous le nom de Trypl. Borreli." (Laveran and Mesnil, 2nd ed. of Trypanosomes et Trypanosomiases, 1912).

A specific description of the genus ensues.

Flagellés à corps allongé, très deformable, souvent courbé en arc, présentant tout le long du bord convexe une membrane ondulante dont le bord épaissi se prolonge en arrière par un flagelle et se recourbe en avant pour aboutir à l'extrémité anterieure d'une masse qui a la grosseur et, jusqu'à un certain point, la structure du noyau principal, situé en regard d'elle, le long du bord convexe. De la meme masse, part un flagelle antérieur libre. Probablement divisions longitudinales binaires égales.

This paper seems to have stimulated the interest of additional investigators, particularly in Europe, for during the next few years several reports of other new species appeared in the literature. Marianne Plehn (1903) described Trypanoplasma cyprini, a trypanoplasm from the carp which was pathogenic to some degree. One of the major morphological differences between this trypanoplasm and Trypanoplasma borreli was the length of the flagella. Trypanoplasma

borreli possessed anterior and posterior flagella of approximately equal length, while those of Trypanoplasma cyprini were of unequal length, the anterior organelle being longer than the posterior.

Leger (1904a and 1904b) described a new species, Trypanoplasma varium, whose host was Cobitis barbatula. The same year (Leger, 1904c) he also extended the host range of Trypanoplasma borreli when he reported this species from the blood of the minnow (Phoxinus laevis). He indicated that the organism was pathogenic in severe infections. Apparently his justification for designating the organism as Trypanoplasma borreli was based entirely on the similarity of morphology between the two flagellates, and not on the fact that the organism from the minnow could infect the red eye (Scardinius erythrophthalmus) or vice versa. Laveran (1904) believed that host specificity must be taken into account and attempted to demonstrate the duality or unity of the parasites. The results of his experiment indicated that cross infection by the flagellate did occur; he concluded that the trypanoplasma of the red eye and the minnow were the same, Trypanoplasma borreli.

Brumpt (1905 and 1906a) described four new species of Trypanoplasma, each from a different host. Trypanoplasma guernei 1905 from the bull head (Cottus gobia) was a large organism, averaging 34 u long, not including the flagella. Trypanoplasma barbi 1906 was found in the blood of the blue bottle (Barbus fluviatilis), Trypanoplasma abramidis in the

blood of the bream, (another large organism 30 u long not including the flagella), and Trypanoplasma truttae from the blood of the brown trout (Salmo fario) (= Salmo trutta).

Up to this time Cryptobia had been found only in land snails, and Trypanoplasma were observed only in the blood of a few freshwater fish. The environment of Trypanoplasma was extended in 1905 by Leger to the alimentary tract of marine fishes when he reported a new species, Trypanoplasma intestinalis from the esophagus and anterior region of the stomach of the host Box boops. Keysselitz (1906) also found a flagellate in the stomach of the marine fish Cyclopterus lumpus, which he described as Trypanoplasma ventriculi.

Keysselitz in 1906 found Trypanoplasma in the blood of the following fish: Perca fluviatilis, Acerina cernua, Lota vulgaris, Barbus fluviatilis, Cyprinus carpio, Carassius vulgaris, Tinca tinca, Abramis brama, Leuciscus idus (Idus melanotus), L. cephalus (Squalius cephalus), L. erythrophthalmus), L. rutilus, Esox lucius and Cobitis barbatula. Keysselitz believed that all the trypanoplasms found in these fish were Trypanoplasma borreli, and that one leech could inoculate several species of fish with the same flagellate. Rodhain (1907) reported a trypanoplasm from the blood of the Congo river fish (Labeo sp.). In 1909 Friedrich published, "Concerning the Structure and Natural History of Trypanoplasma helicis Leidy." He found the organism in the seminal receptacle of Helix pomatia and

said that the animal was the same as that found by Leidy. Here, then, we find an apparent disregard for the genus Cryptobia, as well as Cryptoicus and Bodo, for even though Friedrich stated that the organism in question was that previously described by Leidy, he still resorted to the genus Trypanoplasma of Laveran. Perhaps as Laveran himself said, the genus Trypanoplasma was sanctioned by use. In the same year Crawley (1909) attempted to demonstrate that the organisms from the snail (Cryptobia, Leidy) and those from the blood of fish (Trypanoplasma, Laveran) were congeneric. Crawley obtained the flagellates from Polygyra albolabria and compared them morphologically with drawings of T. borreli, as well as with the definition of the genus Trypanoplasma as given by Laveran and Mesnil. He said,

> This definition evidently covers the parasite of the snail. Further, a comparison of figure 2, a and b, (his drawings of the organisms of the snail) with the drawings of T. borreli, as given by Woodcock (1909, p. 215) shows plainly enough that that animal and C. helicis are congeneric. It may further be added that Friedrich reports longitudinal division, a conclusion that is confirmed by my own material. Hence the name Trypanoplasma must give way to that of Cryptobia.

The reply of Laveran and Mesnil to this argument is not only interesting but also of much substance (Trypanosomes et Trypanosomiases, 1912, page 922). They write in rebuttal:

> Ce flagellé des escargots a été décrit, en 1846, par Leidy sous le nom de Cryptobia helicis; en 1850, par Diesing, sous le nom de Bodo (Cercomonas) helicis et, en 1909, par Friedrich, sous le nom de Trypanoplasma helicis. D'après H. Crawley, le genre Trypanoplasma Lav. et Mesn. devrait être identifié au genre Cryptobia, Leidy, et le nom de Cryptobia qui a la priorité devrait être adopté.

Au point de vue morphologique, Cryptobia helicis a, il est vrai, une grand resemblance avec les Flagellés du sang des poissons pour lesquels nous avons créé le genre Trypanoplasma. Cela ressort notamment de l'étude très complète que L. Friedrich a faite de ce parasite, mois au point de vue biologique on peut relever de notables différences entre ces parasites et on doit se demander si le flagellé qui vit dans le receptaculum seminis et dans les spermatophores de certains escargots, qui est transmis vraisemblablement d' escargot à escargot au moment de la copulation, sans hôte intermédiaire, appartient au même genre que les trypanopl. qui vivent dans le sang des poissons et qui accomplissent une phase de leur évolution dans les sangsues, agents de leur transmission. A côté du genre Cryptobia, il y a place, ce nous semble, pour le genre Trypanoplasma; ce dernier nom est d'ailleurs consacré par l'usage.

In the next decade or so considerable interest prevailed in this area of biological research, and many new species of Trypanoplasma (Cryptobia) were reported from a considerable variety of hosts. The differences of opinion in the taxonomy of the flagellates are observed by the new species described in the ensuing years. It is noted that the majority of European workers adopted Trypanoplasma as the genus, while most American investigators gave priority to Cryptobia. Note also that most of the species are designated from specific hosts; that is, a species specificity prevailed. The number of new species of Cryptobia (Trypanoplasma) described from the blood of freshwater fish has increased steadily over the years. Minchin (1909a) reported a new species, Trypanoplasma gurneyorum, found abundantly in the blood of the pike. The organisms were reported as occurring under two forms, "ordinary and large." The large forms were very scarce, and even though they differed considerably in size from the "ordinary" group, Minchin felt that they were the same species. Trypanoplasma keysselitzi

from the tench (Tinca vulgaris), first observed by Dr. Keysselitz, was also reported by Minchin to occur in two distinct forms. He said:

> One smaller, which, as it is most abundant, I will call the ordinary form; the other, less common, I will refer to simply as the large form. The two forms are easily distinguished in the living state in fresh blood. In preparations they are seen to be distinguished not only by differences in size but also by points of structure.

The point immediately comes to mind that there were apparently two morphologically different flagellates occurring simultaneously; yet, because they occurred in the same host they were considered the same species.

In 1910 Mathis and Leger reported Trypanoplasma clariae from Clarias macrocephalus, caught in pools at Tonkin. The parasite differed from Trypanoplasma borreli by its great size (29-35 u long) and from Trypanoplasma varium Leger and Trypanoplasma guernei Brumpt by the absence of the black pigment. Hence the authors described the organisms provisionally as a new species. This was the first trypanoplasm described from a silurid. Breindl (1911) reported Trypanoplasma magnum from Cobitis fossilis. The first record of the parasite from North America was reported by Mavor in 1915 who found a trypanoplasm in the blood of the common sucker Catostomus commersonii. He designated the flagellate as "probably Trypanoplasma borreli Lav. and Mesn." His justification was that the trypanoplasm found in the sucker was morphologically similar to Trypanoplasma borreli, as regarded size and shape of the body, position and shape of the nucleus, and length of the

flagella. Yakimoff and Schokhor (1917) described Trypanoplasma ninae kohl-yakimovi from the blood of the Turkestan catfish (Silurus sp.). In 1920 Gauthier described Trypanoplasma valentini from the blood of the trout Trutta fario (=Salmo trutta). Brumpt's description of the flagellate was very incomplete as he had seen the organism only in the living state. Ioff, et al (1926) described Trypanoplasma acipenseri as a new species from the blood of the sturgeon (Acipenser ruthenus). In the winter of 1924-25 five of eight fish caught in the Volga River contained the parasite. In September 1926, eight of twelve fish were positive. Ogawa and Uegaki (1927) reported that four species of fish from Formosa were found infected with Trypanoplasma. Present in the blood, the organisms were not designated by name, but the parasites in the different hosts were not identical.

For more than a decade no new species of Trypanoplasma were reported from the blood of freshwater fish. Then Rodhain, in 1942, described Trypanoplasma gandei sp. n. from the blood of the Congo fish Labeo macrostoma. This parasite was characterized by its great size and relatively small parabasal body. The large organisms were 53.5 u long and 15.7 u wide, and the small organisms were 33 u long by 11.5 u wide. Rodhain had discovered the organism in 1907, reporting it then as an undetermined species (Rodhain, 1907). Ostroumov (1949) from the Soviet Union reported Trypanoplasma pseudoscaphirhynchi in the blood of the fish Pseudoscaphirhynchus kaufmanni. The second report of these organisms

from the blood of North American fishes was in 1951 when Katz described Cryptobia salmositica in silver salmon (Oncorhynchus kisutch) and Cryptobia lynchi of the cottids (Cottus rhotheus and C. aleuticus). These fish are native to King County, Washington. Morphological differences and host records verify the validity of these flagellates as new species. In 1954 Davison, et al extended the range of Cryptobia salmositica to the state of Oregon. Seasonal incidence of the infection was reported, as no organisms were found during the summer months. The last published report, to my knowledge, of blood forms of Cryptobia was that of Wales and Wolf (1955) who reported Cryptobia in the blood of several species of wild fishes from the Klamath and Sacramento river drainages of northern California. The organisms, designated as Cryptobia borreli, were pathological in salmon and trout at the Mt. Shasta, California, hatchery. Parasites were found in the skin, blood, ascitic fluid, muscles, and kidneys.

Grasse, in his "Traite de Zoologie", 1952, Tome I, attempted to clarify the generic contest by stating that the genus Trypanoplasma equals Crytobia; in other words, the genera are synonymous. Further, in an attempt to simplify the taxonomy of the genus, Grasse considered all blood-occurring species as Cryptobia borreli. While this is a simple solution to the problem of speciation, I agree with Laveran (1904) that host specificity cannot be ignored.

The taxonomy problems of these organisms are not confined to the blood forms but have been extended to those biflagellates parasitic in the intestine of marine fish. The reports of Leger (1905) and Keysselitz (1906) were followed by Elmhirst and Martin (1910), who described Trypanoplasma congeri from the stomach of a dead conger eel. The organisms were extremely abundant and all stages of division were observed. There was no trace of the parasites in the blood, and 10 additional conger eels examined by Laveran and Mesnil were negative also. Alexeieff (1910) stated that Leger gave an excellent description of Trypanoplasma intestinalis and that his was the species Keysselitz found in Cyclopterus lumpus. Alexeieff also said he could not distinguish Trypanoplasma congeri of Elmhirst and Martin (1910) from Trypanoplasma intestinalis Leger. This same author two years later, however, (Alexeieff, 1912) remarked that Trypanoplasma intestinalis Leger, 1905, Trypanoplasma ventriculi Keysselitz 1906, and Trypanoplasma congeri Elmhirst and Martin 1910, were all one species, Cryptobia dahli Möbius. Möbius (1888) had described "a spindle shaped animal with a flagellum double as long as the body at either pole," under the name of Diplomastix dahlii. Martin in 1913 further complicated the existing taxonomy when he renamed Trypanoplasma intestinalis Leger as Trypanoplasmoides intestinalis Leger. Martin said the organism had three anterior flagella which did not show in stained preparations. Woodcock and Lodge (1921) described a new species, Cryptobia trematomi

from the stomach and intestines of Trematomus bernacchi (Terra Nova Antartic Expedition). They indicated that morphologically there was a distinction from the genus Trypanoplasma in that the undulating membrane is lacking. The posteriorly directed flagellum of the binucleate organism is attached to the side of the body for part of its length; therefore, the genus Cryptobia is more appropriate.

In 1923 when Roskin published on Trypanoplasma dahli (Möbius), the problem of synonomy arose again among Trypanoplasma dahli (Möbius) Roskin (1923), Trypanoplasma ventriculi Keysselitz (1906) and Trypanoplasma intestinalis Leger (1905), as all parasites were found in the alimentary tract. Reichenow (1931), in a general morphological and ecological discussion of parasitic flagellates of the North and Baltic Seas, listed two previously described species of Cryptobia from the intestine of marine fish, Cryptobia dahli (Möbius) Roskin, 1923, (=C. ventriculi?) from Cyclopterus lumpus and Cryptobia congeri Elmhirst and Martin from Conger niger. The latest publication, to my knowledge, on the intestinal forms of Cryptobia, in marine fish, was that Poljansky (1955) who found Cryptobia dahli in the intestines of fish from the Barents Sea, U.S.S.R.

There is one report of a new species of Trypanoplasma found in the digestive tract of Amphibia. Walker (1910) described Trypanoplasma ranae from the intestines of the frog, Rana palustris. Blood forms have also been found in Amphibia. Rankin (1937) found a biflagellate in the blood

of salamanders and said that it so clearly resembled Cryptobia borreli from the blood of freshwater fish that he considered the organism the same species.

Two reports in the literature indicate that the species of the genus Trypanoplasma may be ectoparasitic in nature. Swezy (1916) reported Trypanoplasma carassii as a new species on goldfish (Carassius auratus) and in 1919 she gave a detailed description of the organism. The flagellate was found living on the mucus present on the surface of the fish, and was not found free in the water. Swezy said, "In the structure and arrangement of the nucleus and parabasal body it presents some striking resemblances to Prowazekia to which genus it could properly be assigned were it not for the presence of the undulating membrane." An undescribed trypanoplasm was reported by Wenrich (1931) as ectoparasitic on the gills of the carp (Cyprinus carpio). No organisms were found in the blood. The structure of the flagellate was different from that of Trypanoplasma carassii Swezy (from the skin of the goldfish) and also from Trypanoplasma cyprini Plehn (blood of the carp).

Because the first species of Cryptobia was reported from snails (Leidy, 1846) it is not surprising that additional discoveries of parasitism among Gastropods are recorded in the literature. Kühn in 1911 described the following five species of Trypanoplasma from land snails: Trypanoplasma desertorum from Helix desertorum, Trypanoplasma helicognae from H. pomatia, Trypanoplasma trochearum from

several species of Helix, Trypanoplasma rupestre from H. singula, and Trypanoplasma limnorum from Limnae stagnalis and L. palustrus. In 1914 Collin published on finding Cryptobia carinariae in the snail Carinaria mediterranae. This appears to be the first description of a new species to incorporate the genus as Cryptobia in lieu of Trypanoplasma since Cryptobia helicis was described by Leidy, despite the efforts of Crawley in 1909 to indicate the priority of Cryptobia. In 1923 Fantham found Trypanoplasma isidori in the seminal receptacle of the pond snail Isidora tropica. Here again the possibility exists that Trypanoplasma isidori and Cryptobia helicis are synonymous. While Crawley (1909) attempted to demonstrate that Cryptobia and Trypanoplasma were congeric Matthey (1923) emphasized the distinction between the two genera. In his studies of Cryptobia helicis he regarded the morphology, host distribution, and development as quite distinct from the blood Trypanoplasma; he therefore stated that Cryptobia and Trypanoplasma were not congeneric. Trypanosoma and Trypanoplasma were similar, however, as they are blood dwellers needing an intermediate host. Matthey felt that Cryptobia was more similar to the genus Prowazekia.

Cryptobia (Trypanoplasma) has also been found in a variety of invertebrate hosts. Hesse (1910) described Trypanoplasma vaginalis as an exclusive inhabitant of the female genitals of the leeches Hirudo medicinalis and Aulastomum gulo. The undulating membranes were little

developed. Porter and Fantham (1910) described Trypanoplasma dendrocoeli as a new species from the gut of the turbellarian Dendrocoelum lacteum. The host range of the genus was extended to insects in 1912 when Hamburger found Trypanoplasma gryllotalpae in the intestine of the insect Gryllotalapa vulgaris (Orthoptera). The flagellate ranged in size from 10 to 26 microns long, and the anterior flagellum was double the length of the body. Hovasse (1924) reported Trypanoplasma sagittae from the midgut of a chaetognath (Sagitta bipunctata). This was an extremely large organism, with a body length from 60 u to 100 u in the larger specimen. The use of sections showed the presence of the blepharoplast, myonemes, and basophilic inclusions. The same year Valkanov (1931) recorded Trypanoplasma dendrocoeli Gelei from another turbellarian, Planaria albissima. Sandon (1928) described a new species of free living biflagellate from the soil of New Jersey and Utah. The organism, having no blepharoplast, was placed in the family Cryptobiidae and given the name Dimastigella trypaniformis. It was described as closely resembling the Trypanoplasma. In 1938 an extremely interesting paper was published by Ruinen who reported free living Cryptobia from brackish water in Australia. Two species found in this environment were described as Cryptobia liberia and C. bilata.

The first report of the genus Trypanoplasma (Cryptobia) from the blood of marine teleosts was that of Mackerras and Mackerras (1925. They described Trypanoplasma

parmae as a new species found in the blood from three of nine Australian white ears (Parma microlepis). They report,

> The special interest attached to this parasite is that, while Trypanoplasma (or Cryptobia) has long been known as a parasite of invertebrates, of the alimentary tract of frogs and marine fish, and of blood of freshwater fish, this is, so far as we are aware, the first record of its occurrence in the blood of marine fish.

Their description of the organism, based on dried films, shows Trypanoplasma parmae to be quite different morphologically from the species of Cryptobia found in Great Bay fish. The Australian parasites are generally much broader and shorter than the New Hampshire species. Also, the trophonucleus of Trypanoplasma parmae is situated behind the middle of the body, while the similar structure of the Great Bay organism is located in a more anterior position.

Two other genera are of interest and should be mentioned briefly here because of their relationship to Cryptobia (Trypanoplasma). A new genus Cryptoplasma was erected by Chatton and Blanc (1916a) for an endoparasite of the tick Rhipicephalus sanguineus. Here we see an attempt to combine the genera Cryptobia and Trypanoplasma into one workable genus. However, the "pseudoparasite" Cryptoplasma rhipicephalus was later established to be spermatozoa of the tick rather than a parasite (Chatton and Blanc, 1916b). Duboscq and Rose (1932) studied species of Trypanophis and stated that this genus, found only in Siphonophores, was distinct from the genera Trypanoplasma and Cryptobia by morphology and development cycle.

It is apparent upon reviewing this literature that many organisms are inadequately described and that, in all probability, considerable synonymity exists. While it was the contention of Keysselitz, more recently supported by Grasse, that Cryptobia from various species of fish inhabiting the same body of water are the same species, I feel that a certain host specificity occurs, as well as a specificity displayed by the intermediate host. It must be determined whether or not one species of leech will prey upon several unrelated genera and species of fish living together in an environment.

It is also interesting to observe, in summary, that organisms assigned to the genus Cryptobia (Trypanoplasma) apparently have a rather wide range of environments. The majority of descriptions are those of parasites found in the seminal receptacles of snails and the blood and internal organs of freshwater fish. A few species were reported from the intestinal tract of amphibia, insects, and marine fish, and more recently from the blood of the latter. One species was found in the vagina of a leech; some organisms were ectoparasitic on gills and skin of fish, and some of the flagellates were free living in soil and brackish water.

SECTION III

GENERAL MATERIALS AND METHODS

Marine Fish

The marine fishes used in these studies were obtained by various methods. Most attempts to acquire specimens by means of beach seining with a 1/4 inch common sense minnow seine or bag seine were made shortly after low tide when the incoming currents seemed to provide the greatest number of fish. The following species were procured by this means:

Winter flounder (Pseudopleuronectes americanus Walbaum)

Smooth flounder (Liopsetta putnami Gill)

Striped killifish (Fundulus majalis Walbaum)

Mummichog (Fundulus heteroclitus Linnaeus)

Grubby (Myoxocephalus aeneus Mitchill)

Longhorned sculpin (Myoxocephalus octodecimspinosus Mitchill)

Alewife (Alosa pseudoharengus Wilson)

Striped Mullet (Mugil cephalus Linnaeus)

American Eel (Anguilla rostrata LeSueur)

Usually the larger specimens were obtained by the common method of hook and line fishing which yielded the following species:

Winter flounder (Pseudopleuronectes americanus Walbaum)

Smooth flounder (Liopsetta putnami Gill)

Skate (Raja sp.)

Atlantic tomcod (Microgadus tomcod Walbaum)

Longhorn sculpin (Myoxocephalus octodecimspinosus Mitchill)

Cunner (Tautogolabrus adspersus Walbaum)

Thirty-three specimens were contributed to the study by the U. S. Fish and Wildlife Service, Boothbay Harbor, Maine. These fish, held in the public aquarium, were of the following species:

Winter flounder (Pseudopleuronectes americanus Walbaum)

Longhorned sculpin (Myoxocephalus octodecimspinosus Mitchill)

Shorthorn sculpin (Myoxocephalus scorpius Linnaeus)

Cod (Gadus callarias Linnaeus)

Ocean pout (Macrozoarces americanus Bloch and Schneider)

Sea raven (Hemitripterus americanus Gmelin)

The scientific and common names of fishes used are in accordance with the American Fisheries Society, Special Publication No. 2 - A List of Common and Scientific Names of Fishes from the U. S. and Canada.

Many of the smaller specimens of some of these marine species were maintained alive in circulating sea

water aquaria at the University of New Hampshire. The entire circulation, filtration, and aeration system was contained in a room with a constant temperature of 52° to 54° C. The salt water was completely replaced approximately every six months with natural sea water collected at Portsmouth, New Hampshire. No attempt was made to standardize the salinity, the norm for the system being approximately 32 parts per thousand. Chopped Mytilus edulis, readily obtained from Great Bay area at low tide, was fed to the fish every three or four days.

It was observed throughout the course of these trials that Pseudopleuronectes americanus, Liopsetta putnami, Myoxocephalus sp., Microgadus tomcod, Fundulus heteroclitus, and Fundulus majalis were hardy laboratory species and they were kept with ease in the artificial environment as long as was desired. Other species such as Alosa pseudoharengus and Mugil cephalus displayed a marked inability to be held for any length of time under laboratory conditions.

Freshwater Fish

Common goldfish (Carassius auratus) were purchased at a local pet shop and held in the laboratory in a 10 gallon capacity glass aquarium. The fresh water was changed when necessary and pieces of the plant Elodea were provided for oxygenation. Brook trout (Salvelinus fontinalis) held under similar conditions as the goldfish, were obtained as fingerlings from the New Hampshire State Fish Hatchery at New Durham.

Procurement of Blood Samples

Previous investigators (Novy and MacNeal, 1905 and Minchin, 1909a) have successfully used the following method for procurement of small quantities of blood for smear purposes. The fish is placed on a paper towel and measured if desired. The operculum is held back by means of forceps, exposing the gill filaments from which excess water is removed by blotting with a paper towel. A micropipette, wetted with 1% lithium oxalate, is merely jabbed into the gill filaments. The blood flowing from the wound is aspirated into the instrument by a rubber bulb, either to be released onto a slide for smear purposes or deposited into a drop or two of 1% lithium oxalate for fresh examination. Because fish blood coagulates very rapidly, little damage is done to the fish by the use of this technique; thus it is possible to make repeated bleedings on the same specimen.

When greater quantities of blood were desired, or aseptic techniques compulsory, cardiac punctures were made with sterile micropipettes. In this instance the fish was often stunned by a forceful blow to the head with a blunt instrument to prevent its thrashing about. After the operculum was removed, the entire area was dried with a paper towel and swabbed with 70% alcohol. The end of the rather short sterile micropipette was then forced through the tissue into the pericardial cavity which, in the flounder lying white side down, is just anterior to the pectoral fin, immediately posterior to the gill filaments, in that corner

formed by the gill cavity.

Diagnosis

The first and usual means of diagnosis consisted of withdrawing a drop or two of whole blood from the gills in the micropipette containing a small amount of 1% lithium oxalate. This whole blood was then deposited in a drop of 1% oxalate on a glass slide, the cover slip applied, and the entire fresh preparation examined under low power (10x objective and 20x ocular) for moving organisms. A drop of unoxalated whole blood was also placed on another clean slide at this time and a thin smear made for staining purposes. In the event the fresh preparation proved negative, the stained slide was examined for Cryptobia for approximately one-half hour.

During the course of these studies it became apparent that a satisfactory method of concentrating the organisms was necessary, not only as a diagnostic aid, but as a means of obtaining numbers of organisms for host specificity, immunity, and other studies. One of the methods I used was described by Novy and MacNeal (1905) in their work with bird blood. Several drops of whole blood were quickly added to two or three cc of 1% lithium oxalate in a small vial and thoroughly mixed by a repeated aspiration and discharge of the pipette. The vial was placed under refrigeration where the red blood cells settled to the bottom, leaving a clear supernatant. All the supernatant was removed except for a thin layer covering the sedimented

cells. When this layer was placed on a slide with a cover-slip and examined for the live organisms, the rapid undulating movement of the organism was readily detected.

Another method of diagnosis was described by Yaeger (1960). The procedure was the same as the oxalate method above, plus the addition of 0.1 cc phytohemagglutinin per two cc of oxalate before mixing. The red blood cells were lysed by the material, freeing the organisms. Following differential centrifugation the supernatant was removed and examined for the moving organisms.

One attempt in carrying out the method of diagnosis first mentioned resulted in an excess of whole blood being deposited on a slide in a sub-minimal quantity of 1% lithium oxalate. This mixture was allowed to remain unmolested a minute or two while the thin smear from the fish was being prepared. Upon application of the cover slip, the microscopic examination immediately revealed a very heavy concentration of live, undulating Cryptobia, as well as a rather massive clot of blood which had failed to become defibrinated due to the lack of oxalate. Organisms caught in the clot of blood were noticed escaping into the surrounding serum as coagulation took place. Subsequently several drops of whole unoxalated blood from a known positive fish were placed in a small vial and allowed to clot. A drop of clear serum was removed by means of a capillary pipette, placed on a slide and examined for live moving specimens, as was done with the oxalated whole blood preparation.

Numerous Cryptobia were observed each time the technique was applied with known positive blood. It was observed that further concentration could be accomplished by centrifugation if deemed desirable.

To further test this new technique of concentration and diagnosis, four specimens of the green frog (Rana clamitans) were collected locally, killed and bled. Fresh oxalated preparations were made simultaneously with those using serum, and the results compared. It was interesting to note that the first specimen appeared negative to flagellates according to the whole oxalated preparation, while examination of the serum readily revealed five specimens of Trypanosoma rotatorium. The three remaining specimens appeared positive to Trypanosoma rotatorium, by both methods, although in each instance more organisms were detected by serum examination. Another frog, Rana sylvatica, was examined but proved negative for trypanosomes by both methods.

This serum examination method was also employed in diagnosing an infection of Trypanosoma lewisi in a rat. The animal was killed and whole oxalated blood as well as serum samples were examined for the live organisms.

Even though the trypanosomes were readily detected in the oxalated preparation, the organisms in the serum were present in a far greater concentration, to the extent that the slide appeared, under the low power objective of the microscope, to be a mass of live undulating trypanosomes.

Because the infection in the rat was sufficiently heavy to make a positive diagnosis easy by means of the oxalated preparations, a period of three weeks elapsed, when the rat was bled again. The numbers of flagellates had decreased considerably, but their detection was easily made by the serum examination method.

Five newts (Diemictylus viridescens) were examined for blood flagellates by placing a drop of whole blood from each animal on a slide, applying a cover slip, and waiting for the serum to separate out. Trypanosomes were readily detected in four of the five specimens, the fifth and smallest being apparently negative.

In as much as this technique (of allowing the whole blood to clot and collecting the serum) seemed to concentrate the organisms it was employed in the trials involving host specificity, immunity, and division studies. Blood from positive fish was drawn in considerable quantities, often by direct cardiac puncture, and allowed to clot. The serum containing the organisms was then easily injected by means of a syringe into other fish or animals. I also observed that thin smears of serum containing the concentrated organisms could be stained in the same manner as whole blood preparations. Care was taken to avoid thick smears, for the lack of blood cells gave the appearance of a thin preparation. Thick smears stain too heavily and obscure the organisms.

Stains of Blood Smears

Giemsa stain was used frequently for diagnostic

purposes as the method seemed to lend itself most readily to the conditions of the study. Thin blood smears were made from individual fish in the field, air dried on location, and then transported back to the laboratory for fixing and staining. Such a procedure could not easily be done with techniques involving the use of osmium tetroxide. The fish had to be transported to the laboratory in a live condition when this method was employed. (The exact procedures for this and ensuing stains is found in Appendix I).

It was pointed out by Minchin (1909a) that shrinkage and distortion occur to a considerable extent when the films are immediately air dried prior to fixation. For that reason he killed the organisms before drying by placing the slide, immediately after smearing, into 4% osmic acid fumes for 30 to 60 seconds. Then, with or without air drying the films were placed in 100% alcohol. The smears were stained with Giemsa's stain. Minchin indicated that with this procedure the organisms tend to stain too darkly and to become opaque. Consequently a much shorter staining period is required when osmic fumes are used. I found that staining with Giemsa for two minutes seemed to produce the best results. The flagella were clearly distinguishable though the cytoplasm remained somewhat opaque. While the technique was not employed to any extent in these studies, perhaps it does offer a means of staining rapidly with osmic acid. I consider Taliaferro's method to be much superior, though more time is involved in carrying out the procedure.

Though other modifications of Minchin's techniques were attempted, differentiation of the nucleus and blepharoplast from the cytoplasm was very difficult and the method was discarded.

Taliaferro (1923) employed much the same procedure as Minchin when he stained smears exposed to osmic fumes with Giemsa, with the important addition of hydrogen peroxide as a bleaching agent. This prevented the smear from staining too darkly with the result that the cytoplasm stained a light pink, with nucleus, parabasal body and flagella staining a darker bluish red color. As results with Taliaferro's technique were consistently satisfactory this method was used throughout the rest of the investigations. Taliaferro showed significant variations in measurements between air dried Giemsa stained films and osmic vapor killed preparations. Therefore I made two sets of measurements of the organism studied, one using air dried film and one with the osmic acid technique.

Most iron hematoxylin stains require overstaining followed by decolorization. Generally the differentiation is controlled by microscopic examination of the preparations. Johnson's technique as outlined by Craig (1948) is comparatively rapid and required no destaining procedures. Therefore I attempted to adapt it to blood flagellate use. The results were extremely disappointing. Coloration was much too dark to discern any internal morphology and flagella and cell outline were very indistinct. As destaining

appeared necessary I attempted Minchin's (1909) modification with Heidenhain's iron-hematoxylin. The flagellates were killed in osmic fumes and the wet smears were then fixed in Schaudinn's fluid (Appendix I). Successful use of this technique depends on exact extraction of the stain, and I obtained satisfactory preparations after several tries.

Attempts were made to apply Feulgen's nuclear reaction to blood smears positive with Cryptobia. The organisms were very difficult to detect in the smear as the flagella and cytoplasmic wall were barely visible. Although modifications were tried, the results were generally unsatisfactory. Best results were obtained when the parasites were killed in osmic fumes and the smears air dried before fixation with Schaudinn's fluid. This technique was not used routinely.

Kozloff (1948) used a protargol technique routinely in his work with Cryptobia from land snails. Application of the method to Cryptobia positive blood smears produced encouraging results as regards the flagella, kinetoplast and nucleus. While the procedure was not used routinely, it appears to be desirable for staining nuclear and flagellar structures.

Leeches

Inasmuch as Brumpt (1906b), Laveran and Mesnil (1901), Keysselitz (1906), Robertson (1911) and others have shown the role of Hirudinea as intermediate hosts in Cryptobia (Trypanoplasma) infections of freshwater fish, attempts

were made to obtain leeches from the same environment as marine fish known to be infected with the flagellate.

The first endeavor was to examine the flounders obtained by seining. These fish and pieces of algae collected with them were examined carefully upon removal from the seine. Even though 90% of the flounders caught in this manner may have been positive for Cryptobia, not once did I find any leeches on the fish. Vegetation likewise proved to be negative, as did the sea water used in transporting the live fish to the laboratory aquaria.

Another attempt to obtain leeches was made by collecting bottom samples with a long handled bottom net. The net was dragged fairly rapidly along the bottom mud and then the contents were examined very closely for the presence of the desired invertebrates. All collections proved negative, however.

The first leeches I collected were those found on small flounders caught by ice fishermen on hand bob lines. As the small fish were pulled from the water, leeches were occasionally found to be attached to them. As the ice fishing season is a relatively short one, hand line fishing from a boat was resorted to the remainder of the year. This indeed proved to be the most fruitful method of obtaining the animals, and the majority of leeches collected were acquired by this method. I observed that often leeches would be found adhering to the sides and bottom of the boat as they had become detached by the violent contortions of fish

when removed from the water. Because of these contortions it is possible that many flounders, perhaps even the majority, are parasitized by these Hirudinea, but the leeches leave the host before their presence is observed. In the practice of seining, the parasites have ample opportunity to be shaken loose from their hosts before being detected.

The leeches were taken from the fish and placed in vials of sea water for removal to the laboratory. Following microscopic examination to determine if Cryptobia were present, the leeches were placed in vials of fresh sea water and kept under refrigeration at approximately 40° F (4.5° C). The water was changed every one or two weeks. I was able to hold leeches in this manner for over three months. Even though no food materials were supplied during this term of captivity, no leeches died from natural causes under these conditions.

Leech Whole Mounts

In preparing leeches for whole mounts the first step involved was that of relaxation. Warm sea water, heated to 60° C was tried, but the animals immediately died in a state of contraction. A few grains of tobacco in three or four ml of sea water was effective over a period of several hours. The material which gave the best results was a compound known as MS 222 (Tricaine methanesulfonate, by Sandoz Pharmaceutical). This chemical has been used very effectively in great dilution as anesthesia for fish and lower aquatic invertebrates. Using a dilution of 1:2000,

I found most leeches fully relaxed following ten to 15 minutes immersion in the solution. Because of its rapidity and effectiveness, MS 222 was used.

Fixation was accomplished with either Bouin's or Demke's fluids; both were satisfactory (see appendix). When using Bouin's fixative, repeated 70% alcohol washes were used until all yellow color had disappeared. With Demke's fixative two or three changes were employed. Also, a small amount of eosin (1-2 drops in 40 cc alcohol) for two minutes was utilized as the only stain. This was usually added to the 85% alcohol.

In those instances where the leeches were rather large, a cover glass was laid over them during the period of fixation. This tended to flatten the animals somewhat, making permanent mounting a little easier.

When serial sections of leeches were prepared, relaxation with MS 222, fixation with either Bouin's or Demke's fluids, and dehydration were the same procedures as those used in the whole mount preparations. A series of frontal, longitudinal, and transverse sections were prepared and stained with Delafield's hematoxylin.

Other Animals

Frogs. The frogs used in the host specificity studies were Rana pipiens, purchased from a biological supply house. These animals were held on the freshwater table of one of the aquaria rooms at the University of New Hampshire. Inasmuch as the frogs were kept only for a

period of approximately two weeks, no attempt was made to feed them.

Mice. The mice used in the cross infection studies were Mus musculus. These were obtained from a supply of this species at the University of New Hampshire. The animals were maintained in plastic mouse cages and were fed Purina "laboratory chow".

SECTION IV

LIFE CYCLE STUDIES

Invertebrate Hosts

The Leech As a Means of Cryptobia Transmission.

The role of leeches in the transmission of trypanosomes and trypanoplasmas has been clearly demonstrated by Brumpt (1906b) and Robertson (1911). Therefore leeches were suspected of transmitting this new species of Cryptobia to the flounders of Great Bay, N. H. This suspicion was strengthened when Bullock found a leech attached to a small flounder. The annelid was crushed, smeared on a glass slide, fixed and stained with Giemsa. Numerous Cryptobia were present and appeared identical to those in the flounder blood (Plate 1, 6 and 7).

When these studies were begun there was no indication that leeches would be difficult to obtain. My first two specimens were found on February 9, 1960, attached to the dorsal side of a small Cryptobia-positive flounder caught through the ice at Great Bay, N. H. I took the small, olive-colored worms back to the laboratory, mounted one in a few drops of sea water on a slide and examined the animal with the low power objective of the microscope. The examination was frustrating as the leech moved constantly, even crawling out from under the cover slip. After a few minutes

the animal ceased its body movement and its internal structure could be seen with sufficient clarity. Numerous elongate undulating organisms were easily detected in the intestine. No blood cells were seen. The leech was returned to fresh sea water, but failed to recover in a twenty-four hour period. Another examination the following day failed to reveal movement of the parasites inside. I did not see any organisms in the second leech, so both specimens were fixed in Bouin's fluid. Sectioning the leeches was difficult as the animals were brittle and frequently the tissues fell out of the paraffin.

The third leech, obtained June 20, 1960, was found dead in the bottom of a pail containing flounders caught on hand lines. Neither Cryptobia nor blood cells were seen in the worm. The animal was fixed in Demke's solution, dehydrated, embedded in paraffin, and sectioned. The results were very satisfactory when stained with hematoxylin-eosin.

I was unsuccessful at finding leeches on flounders obtained by seining, bottom scrapings, or on vegetation. The most fruitful means of acquiring the worms was fishing for the parasitized flounders with hook and line. This method yielded a total of 31 leeches during the summer of 1960. Seventeen of these specimens were extremely small. In all instances the animals were relaxed in a 1:2000 dilution of MS 222 (Tricaine methanesulfonate) and then examined for internal flagellates. Only three of the 31 leeches were positive. All leeches, with the exception of

the first two, were taken from larger flounders, 15 cm. or over. The incidence of Cryptobia infection in these larger fish was low.

On one occasion, four large leeches were found on one flounder. Often the leeches, easily released from the host, were discovered in the pail containing the fish.

On August 2, 1960, two leeches were found on the same flounder (L. putnami, 17 cm.) in Little Bay, N. H. Twenty other flounders and 12 tom cod did not appear to have leeches attached to them. These two worms appeared negative when examined in the laboratory and were held in refrigerated sea water.

One week later a heavily infected flounder was put into a bowl of clean sea water and both leeches were placed on its dorsal side. The smaller of the two refused to attach; with its posterior sucker the other leech attached itself immediately to the fish. Four hours later the worm was removed, anesthetized with MS 222, and examined for Cryptobia. Neither organisms nor blood cells were seen, so the leech was returned to refrigeration. The following day the small leech appeared dead. The larger leech again refused to attach itself to a flounder, and was returned to sea water for seven days. At that time another opportunity to feed was rejected. Another attempt to infect negative leeches from positive flounders was made two weeks later. A large negative leech, taken from flounders caught seven days previously, was placed on the ventral side of a positive

smooth flounder (8.2 cm.). The worm attached itself readily and was left to feed overnight. Twelve hours later it was still attached, though it had changed its location on the fish. Examination of the leech following relaxation in MS 222 for ten minutes failed to reveal blood cells or Cryptobia. I could see no evidence that the animal had fed on the flounder. The leech did not revive when placed in fresh sea water. It was fixed in Demke's fluid and later sectioned. Another leech, isolated from flounders seven days, would not attach when placed on a positive host.

Robertson (1911) stated that leeches (freshwater species) require at least ten days between feedings and generally much longer. Therefore, I made a series of attempts to have leeches feed on flounders, using for each trial leeches starved for successively longer periods of time. For the next trial I used a small leech that had been in isolation for 21 days. The worm attached itself readily and remained for ten hours. Microscopic examination showed no indication that feeding had occurred, and no organisms were seen. Another attempt was made with a leech that had been starved for 30 days. The worm attached itself readily with its posterior sucker when it was placed on the dorsal side of a heavily infected flounder. The next day (twelve hours later) the leech was swimming freely in the water, no longer fastened to the fish. Examination failed to reveal any parasites. This trial was repeated with a large, apparently negative leech obtained August 30. Thirty days later

the worm was placed on a positive flounder and remained attached 12 hours. No Cryptobia were found in the leech during a 20 minute microscopic examination.

As all attempts to have leeches feed on flounders had apparently been negative thus far, Dr. J. H. Barrow (1960) suggested to me that one possible reason for this failure to feed may have been due to the warm temperature of the water. These 12 hour trials had been conducted at a room temperature of about 70° F. Two large leeches, caught August 30, were placed in a vial of sea water, which in turn was put in a portable ice chest half full of ice. A bowl of sea water containing a positive flounder was also chilled in the same manner. Both leeches and host were pre-chilled for 12 hours at a water temperature of 3° C. before I placed the leeches on the fish. No light was allowed to enter the chest during the trial. Both worms attached immediately to the flounder with their posterior suckers. When the leeches were removed, one eight hours later and the other 24 hours later, they were firmly attached with both anterior and posterior ends. Microscopic examination, however, failed to reveal the presence of either blood elements or Cryptobia in the intestines of the annelids. Both leeches refused attachment to other fish immediately following the examination. Two days later I repeated this experiment using leeches obtained August 16, or a total of 58 days of starvation. Neither worm would attach itself to the fish. They were left in the water with the flounder, and twelve hours

later both specimens were still swimming freely.

As attempts to infect negative leeches from positive fish consistently ended in failure, I tried a reciprocal experiment to infect a negative fish with a positive leech. The annelid, taken from a flounder caught in Little Bay on August 26, 1960, showed many undulating organisms in the mid-intestine when examined under the microscope. This leech had been starved for five weeks. The flounder was taken in a seine at Duxbury, Mass., where Cryptobia have not been found to date. This fish had been negative to three blood examinations, the last completed one hour before the positive leech was placed on the fish. The leech attached itself to the flounder immediately with both suckers, giving the appearance that it was feeding. The annelid was removed from the fish ten hours later and put in the refrigerator for the night. I failed to find any Cryptobia in this leech when it was examined twelve hours later. There was some debris in the intestine, but no red blood cells were seen. The flounder was first examined for Cryptobia 45 hours after removal of the leech. The results were negative. Successive examinations were made five, ten, and 15 days later, but no Cryptobia were seen. I cannot explain the case of the missing organisms. It does not seem likely that the fish could have been refractory to infection. Robertson (1911) states, "at higher temperatures the leech is able, during the active period of secretion, simply to digest the trypanosomes." It seems unlikely, however, that

this occurred with Cryptobia.

Another attempt was made to demonstrate conclusively the role of the leech in the transmission of this species of Cryptobia. Leech number 21 was taken from a flounder August 29 and had been starved until the time of this trial, January 9, 1961, a total of 19 weeks. I saw no organisms in the worm a week earlier. The leech fastened itself to the positive flounder immediately with both suckers; then the bowl containing the specimens was refrigerated overnight. Fifteen hours later I was amazed and delighted to find the stomach and cecae at the posterior end of the worm abounding with undulating organisms. The great majority of the parasites were attached to the stomach and cecal wall. Very few were free in the lumen. This event was in agreement with Robertson (1911) who found that trypanoplasma regularly attach themselves to the wall in the leech. Robertson also stated that trypanoplasma begin to divide four to five hours after ingestion by the leech. Her observations were similar to mine, the presence of many organisms just 15 hours after commencement of feeding.

Because I was unable to detect dividing forms of the parasites in the leech, and also because I did not see any red blood cells in the digestive tract of the worm, a shadow of doubt crossed my mind and I wondered if perhaps the leech was infected before being placed on the flounder, the organisms having escaped discovery during the earlier examination (All leeches were examined for Cryptobia once

when obtained and again prior to use in transmission trials). The leech was very inactive following this discovery and I feared that the animal would die; therefore, I cut it into pieces in Ringer's solution and injected the inoculum into two negative flounders and one Fundulus. The smallest flounder, 5.5 cm. long, was found dead in the aquarium about 40 hours later. The heart and gills were crushed and examined but no organisms were seen. The surviving flounder and Fundulus were examined three, five, nine, and 15 days post-inoculation, but negative results were recorded each time.

There were two possible explanations for the failure to demonstrate the completion of the life cycle of this species of Cryptobia, that from the leech to the flounder. The first is the short time the organisms were allowed to remain in the leech before the attempted transfer to the flounder. Robertson (1911) found that development in the leech took several days before the organisms were transferred back to the fish. On this basis the inoculation of my infected leech was done much too early to allow this development to occur. The following quotation from Robertson's papers may also be pertinent: "It may be mentioned in passing that leeches sometimes suck lymph instead of blood. Trypanosomes in an infected fish seem to be as numerous in the lymph as in the blood." I have never seen definite red blood cells in any of the leeches I have examined from Great Bay. I saw no erythrocytes in the smear of the Cryptobia-positive leech found by Bullock but in this instance the

time of the last feeding by the leech is unknown.

The second possible explanation for the failure to demonstrate transmission is that it now appears the surviving inoculated flounder was refractory to Cryptobia for some unknown reason. Five days following the last Cryptobia examination, I inoculated this fish intraperitoneally with 0.2 cc of Cryptobia positive serum. No flagellates were found two and five days postinoculation. While this unusual situation may have occurred with the flounder, it does not explain the inability of the Fundulus to become parasitized. A later inoculation of this fish with positive serum was successful, demonstrating susceptibility.

I have planned further work in this study of transmission and developmental stages of the organisms in the leech. I feel the above study, even though not carried to completion, will serve as a foundation for further investigations in this area.

During the course of my study with the leeches, it seemed to me that all these annelids were probably the same species. I have had no previous experience with this group of animals, but I was fortunate in securing the interest of Dr. Marvin Meyer, University of Maine, an authority on Hirudinea. He very kindly spent many hours with me and my materials, attempting to place the leech in its proper taxonomic niche. He concluded that these leeches of Great Bay, N. H., serving as intermediate hosts for the species of Cryptobia being investigated, belong to an undescribed

species, or possibly one of the inadequately described forms named by Verrill. There is even some question as to which of several genera of Pisciocolidae the leeches may belong.

The present state of leech taxonomy is in a state of confusion, and while it may be tempting to establish a new genus and species of Piscicolidae for this leech of Great Bay flounders, such a procedure may prove unncessary and only serve to add to the taxonomic turmoil in the future. Following Dr. Meyer's suggestion, I am assuming my material is identical with Pontobdella rapax Verrill, 1873, (Plate 4). I realize, however, that Pontobdella rapax is not a valid species today, and the leech which Verrill described under this name was assigned to the genus Piscicola by Moore (1898), and to the genus Ichthyobdella by Sumner, Osburn, and Cole in 1913. As Ichthyobdella is synonymous with the preferred Piscicola, I am using provisionally for the purposes of this study the name Piscicola rapax Verrill, 1873, (Moore, 1898). I have ample material of both serial sections and whole mounts that can be made available to anyone interested in an intensive study of the family Piscicolidae.

Because all the leeches taken from Great Bay appeared to be the same species, I wondered if other species of leeches were capable of transmitting the Cryptobia of flounders. Two leeches from rock gunnels (Pholis gunnellus) caught at Sea Point, Kittery, Maine, appeared negative for Cryptobia when examined as whole mounts and stained smears. Two unidentified Hirudinae were found attached to a sculpin

caught in July at Kent Island, New Brunswick, Canada. Approximately five weeks after removal from the sculpin, I placed both leeches on the dorsal side of an infected flounder. Both worms refused to attach to the fish over a period of seven hours. Negative results were likewise obtained with Fundulus.

Another trial of this nature was conducted using freshwater leeches. Two Fundulus, positive for Cryptobia, were first placed in 50% sea water. One hour later they were removed to 25% sea water, and at the end of another hour they were changed to entirely fresh water. I then attempted placing three fresh water leeches on one Fundulus and two more of the worms on the second Fundulus, but the fish were much too active to cooperate. Both Fundulus were anesthetized in 1:2000 MS 222 and washed quickly in two different rinses of fresh water to remove the drug. Then I put the leeches on the immobile fish. Two of the worms attached for a couple of minutes and then slid off. The other three specimens refused to affix themselves at all over a period of four hours. These leeches had been held under refrigeration about three weeks prior to the trial. The results of these two experiments perhaps demonstrate the specificity of leeches for certain hosts, but no information was obtained on their ability to harbor Cryptobia.

Argulus As a Means of Cryptobia Transmission.

During the course of these studies, hundreds of flounders were caught, yet leeches were found on very few of the fish.

But the majority of the flounders had from one to several specimens of Argulus attached to them. Minchin (1909a) had a similar experience with the freshwater fish he studied, and he mentioned how easily Argulus may be studied under the microscope. The animal is transparent and all organs, intestines, and blood circulatory system are clearly seen. Minchin was unsuccessful at finding flagellates in any of the numerous Arguli he examined. I examined dozens of these animals taken from Cryptobia positive flounders, and saw neither flagellates nor red blood cells. I conclude that Argulus plays no role in transmitting Cryptobia to flounders of Great Bay, N. H.

Oral Ingestion as a Means of Cryptobia Transmission. While the role of leeches in the transmission of Cryptobia has definitely been shown, the possibility had to be investigated that the fish could become infected by simply ingesting the flagellates with their food. This possibility becomes quite distinct when one considers that Ruinen (1938) described two species of Cryptobia as free living in brackish water (Cryptobia liberia and C. bilata). Both Minchin (1912a) and Laveran and Mesnil (1912) mentioned that in nature animals may become infected with trypanosomes by devouring other animals containing the flagellates. Heisch (1952) reported that bush babies (Galago) became infected with T. gambiense and T. rhodesiense from eating infected rats. Minchin (1912a) said that intestinal trypanoplasms are probably acquired when the host accidentally swallows cysts or other resting stages

of the parasite, passed from a former host. I conducted two brief experiments pertaining to this matter.

The first trial was carried out to see how long the flounder species of Cryptobia would live in sea water. Two tenths of one cubic centimeter of sea water was added to 0.1 ml of Cryptobia positive serum, and the sample was immediately examined microscopically. The reduction of undulations of the flagellates was dramatic. Some organisms became motionless in about three minutes while others continued spasmodic movements for more than 30 minutes. Additional Cryptobia placed in sea water only, immediately showed infrequent undulations, and the majority of organisms appeared dead by 5 minutes, though some moved slowly for 15 minutes or more. I repeated the trial in a slightly different manner, using controls. Two drops of Cryptobia positive serum were placed on a slide and the cover slip was sealed with vaseline. (Further reference to this technique is on page 57). On a second slide, one drop of positive serum was mixed with one drop of sea water, the cover slip sealed as before, and both preparations refrigerated. When both slides were examined microscopically 24 hours later, many normal, live, rapidly moving Cryptobia were seen beneath the coverslip. All flagellates were dead, however, in the sea water-serum preparation. No live organisms were seen on the entire slide, though many dead flagellates were easily observed. The results of these trials demonstrate the detrimental effect of sea water on the species of Cryptobia from the

blood of the flounder. It does not appear that these flagellates could ever be free living for any length of time.

Even though the results of these first trials showed that oral infection probably did not occur, a second experiment was conducted to observe the effects of force feeding Cryptobia to negative flounders. I also felt that the information obtained would be of value in the taxonomic study of the parasite, to see if the intestinal species of Cryptobia was also capable of living in the blood of the host, and reciprocally.

A small smooth flounder, negative to three blood examinations, was anesthetized with MS 222 and then force-fed approximately one-half ml of Cryptobia positive serum. The organisms were administered by a capillary pipette, forced through the oral opening past the pyloric valve until I felt, with my fingers, the end of the instrument in the intestines. I did not observe regurgitation of the fluid when the pipette was withdrawn. After inoculation I returned the fish to MS 222 a few minutes before transferring it to sea water. Blood smears from this fish were examined three, six and ten days postinoculation. Results were negative each time. I also failed to find Cryptobia in the intestinal and stomach contents of the flounder following the last blood examination.

I conducted a second trial of this nature using a small gelatin capsule filled with Cryptobia positive serum. A flounder, negative to three blood examinations, was

anesthetized in MS 222. Then I forced the capsule through the esophagus into the pyloric area and returned the fish to MS 222 for another 15 minutes. Regurgitation of the capsule did not appear to occur, even when the fish was returned to fresh sea water in the aquarium. Repeated attempts failed to have the fish consume the capsule on its own accord. Blood examinations five and six days postinoculation were negative for Cryptobia, and no organisms were found in the stomach or intestines seven days after the capsule was fed the fish. The results of these trials show that the oral route cannot be considered as a means of transmission of this species of Cryptobia. The trials also show that Cryptobia of the blood of the flounder will not maintain themselves in the alimentary tract of the same host.

Vertebrate Hosts

Studies of Division in the Vertebrate Host. The fate of Cryptobia (Trypanoplasma) after inoculation into the vertebrate host by the leech has not been clearly defined in the literature. Laveran and Mesnil (1901) in their original paper describing Trypanoplasma borreli n. g., n. sp., state that they have never seen forms of division in these hematozoans. They postulated that there is a rather short period of multiplication, as in Trypanosoma lewisi, after which the parasites persist in the blood without further division. In 1904, however, Laveran and Mesnil observed several forms of division in the blood of the red eye

(Scardinius erythrophtalmus) infected experimentally with Trypanoplasma borreli. The centrosome divides first, then the flagella divide in two parts. A figure depicting this process is presented on page 395 of Trypanosomes et Trypanosomiases, Vol. 1, 1904. Plehn (1903), commenting on the absence of dividing forms in Trypanoplasma cyprini, is of the opinion that either the dividing period occurs very rapidly or it does not occur in circulating blood. Laveran and Mesnil (1904), however, upon examining a preparation of Trypanoplasma cyprini sent them to M. Plehn, found "several elements, evidently in the course of division." The organisms displayed two nuclei and two centrosomes. Keysselitz (1906) described the reproduction of Trypanoplasma as a sexual process, with male and female gametes destined to conjugate in the leech, but his hypothesis has received no support. Minchin (1909a) strongly suspected some form of multiplication of trypanosomes in the internal organs of fish, occurring in the first few days following inoculation as in Trypanosoma lewisi. He notes, however, "The absence of fission-stages in the blood of fish infected naturally is very striking." Robertson (1911) conducted a comprehensive study of the transmission of flagellates in the blood of fresh water fishes, yet never saw division stages of trypanosomes in the vertebrate host. More recently, Baker (1956) failed to detect multiplication of Trypanosoma avium Danilewsky in the blood of birds. Katz (1961) reports finding rare dividing forms of Cryptobia salmositica in salmon.

In the course of these studies I have observed thousands of Cryptobia from the blood of flounders in both fresh preparations and stained smears, yet I have never seen the flagellates reproducing by division or any other means. However, I have seen organisms which, at a superficial glance, did appear to be dividing (Plate 2, 4 and 5).

Because of the strong suspicion that multiplication did occur in the blood of flounders, yet dividing forms were not to be found, I conducted three trials to determine if reproduction of this species of Cryptobia occurred shortly after the fish became infected, as is the case with Trypanosoma lewisi in rats. For the first trial I used three flounders, each negative to three blood examinations. Each fish was inoculated intraperitoneally with 0.1 cc of heavily infected serum. The first fish (L. putnami, 8.0 cm.) was bled 24 hours post inoculation. Parasites were detected neither in a fresh preparation nor in a Giemsa stained smear. This flounder was next bled 48 hours after receiving the flagellates but no dividing forms were seen in either fresh or stained smears. Sixty hours post inoculation, fish 2 (L. putnami, 5.5 cm.) was bled and killed. Many Cryptobia were found in the fresh smear, and it was at this time that I saw a live Cryptobia undergoing what possibly could have been division. The movement of the organism was ameboid with no undulations. As no stains were present, nuclear division was not observed. It seemed as though the organism was splitting down the center, appearing in the form of a V.

I thought I detected flagella on each anterior portion of the parasite, as well as the usual trailing flagellum. I then lost the organism in a field heavy with erythrocytes and rapidly flowing from under the cover glass. I examined every parasite on the stained smear with the oil immersion objective, a process that took several hours, yet I did not see any forms suggesting reproduction. Fish three of this trial (L. putnami, 5.0 cm.) was bled and killed 72 hours after injection with Cryptobia. The resulting infection was very light and no dividing forms were seen, either in the fresh or smeared stains. The first fish (L. putnami, 8.0 cm.) was bled again 84 hours post inoculation. There were many Cryptobia present in the fresh blood preparation but none of the organisms gave the appearance of division comparable to that just described from fish two. No multiplying forms were seen when each parasite on the stained smear was examined with high magnification.

Two negative flounders were used in the second attempt to demonstrate Cryptobia division. Each fish received one intraperitoneal injection of 0.1 cc positive serum. Permanent smears were completely examined at magnifications of 860 and 1904 x. One flounder (L. putnami, 8.5 cm.), bled 24 hours post inoculation, showed many Cryptobia in the fresh blood smear, but no dividing forms were seen in the stained preparation. The second flounder (P. americanus, 7.7 cm.) was bled 48 hours following the injection of parasites. I saw few organisms in the fresh

smear and I did not detect dividing forms on the stained slide. This fish was found dead the following day, but a brain smear was negative for Cryptobia. The first flounder was bled again 60 hours post inoculation and dividing forms were not apparent in either the fresh or stained smear. Another examination of the blood from this fish 72 hours post inoculation failed to reveal any dividing forms, though many Cryptobia were present.

I used two flounders in the third attempt to demonstrate Cryptobia multiplication in the blood of its vertebrate host, L. putnami (7.5 cm.) and L. putnami (8.2 cm.): Both fish were inoculated intraperitoneally with 0.1 cc. of Cryptobia positive serum. When these flounders were bled 49, 60, 86 and 120 hours post inoculation, no reproducing forms were seen in either fresh preparations or stained smears.

In the above described attempts to demonstrate multiplication of Cryptobia in the blood of flounders, I noticed an apparent increase in the number of flagellates appearing in the blood of the inoculated fish 48, 60, and 72 hours following injection. As no dividing forms were seen, what was the explanation of the perceptible increase of organisms in the blood?

I then injected 0.1 cc. of positive serum via the peritoneal cavity of two negative flounders. Twenty four and 48 hours later I killed the fish and examined the peritoneal fluid for Cryptobia. I found many organisms in the

peritoneal fluid of each flounder, though forms of reproduction were absent. As the flagellates can live at least 2 days in the peritoneal fluid, their period of entrance into the blood and lymph vessels of the living membranes may extend over many hours. Obviously all the parasites do not enter the circulatory system at the same time.

Robertson (1911) demonstrated on glass slides that when water was added to fish blood containing trypanosomes the flagellates divided after six to nine hours. For my first attempt at this procedure I mixed on a slide one drop of heavily infected flounder blood with one drop of distilled water, sealed the cover slip with vaseline and refrigerated the preparation. Red blood cells were being lysed at this time. When I examined the slide 16 hours later, all Cryptobia were dead. I repeated the procedure a second time, making two preparations of blood plus much smaller drops of tap water and one slide of blood plus a drop of one per cent lithium oxalate. Eighteen hours later all organisms in the oxalate preparation were dead, but the organisms were very lively on the two slides containing blood and tap water. I did not see any dividing organisms. These two slides were examined every 12 hours for nine days, when many motile bacteria appeared and the Cryptobia gradually became less active and died. I saw no reproducing forms at any time, though rosette clumping of the parasites was seen frequently.

I made another attempt to demonstrate dividing forms of Cryptobia on slides, using two drops of positive serum

with no water or oxalate. The cover slips were sealed in vaseline and the slide was refrigerated in a household refrigerator. The flagellates remained lively for 6 days, then began to die rapidly as bacteria became more prominent. At the end of the seventh day all Cryptobia were dead. I was able to maintain Cryptobia as long as 12 days in other trials using this method.

Because of my unsuccessful attempts to observe multiplication of the flagellates in these slide preparations, I wondered if perhaps division had occurred, but it was not apparent to me. Therefore I repeated the above procedure with 4 slides made from heavily infected flounder blood and very small drops of distilled water per slide. At varying intervals I removed the cover slip and smeared the drops of serum on clean slides, exposed the organisms to osmic fumes 30-60 seconds, after 19 hours incubation; the second slide, 27 hours; the third slide, 41 hours; and the last slide was prepared after 62 hours. The preparations were stained with Giemsa and then examined, but I failed to find any multiplying forms.

To further demonstrate that Robertson's method of propagating trypanosomes on slides was not applicable to my species of Cryptobia, I prepared two slides with positive serum and a small drop of water as described above. Slide one contained 25 live Cryptobia and slide two held nine flagellates. The preparations, held under refrigeration were counted daily. The number of organisms on each

slide remained constant for three days. On the fourth day only five live Cryptobia were found on slide one, and five live organisms were on slide two. No multiplication occurred in either preparation.

Longevity Trials. Because of my consistent inability to observe forms of multiplication in the vertebrate host or in glass slide cultures, the question became pertinent as to how long the fish remained infected when denied the opportunity of reinfection by leeches. On June 15, 1960, I examined the blood of four flounders held in the circulating sea water tanks. Each of these flounders was positive for Cryptobia when caught through the ice of Great Bay on February 9, 1960. Flounders 11 (L. putnami, 9.8 cm.) and 12 (L. putnami, 0.5 cm.) still retained the infection, while fishes 13 (P. americanus, 11.8 cm.) and 14 (L. putnami, 10.3 cm.) appeared to have lost their flagellate parasites. Fishes 11 and 12 have been bled at monthly intervals since.

A reduction in the number of flagellates was noticed in flounder 11 at six months, and eight months after the fish was caught no flagellates were seen. The flounder has remained negative since. Fish 12, however, has maintained its infection to the present time, a period of 13 months.

On June 17, 1960, ten heavily infected flounders, taken from Crommet Creek, were added to the aquarium. All specimens were carefully examined for leeches but none were found. The fish ranged in size from 7.0 cm. to 8.6 cm.

Each flounder was examined at monthly intervals; all of these small flounders have remained heavily infected to this writing, a period of nine months. Repeated bleedings have been responsible for the death of six specimens, however, because of loss of blood and damaged gills. Two of the survivors are in good flesh and the other two fish appear quite thin. At the time of the seventh monthly bleeding, one extremely thin flounder appeared nearly dead prior to the examination. I saw no live Cryptobia in the blood of this fish, but dead flagellates abounded. I could see their flagella very clearly. The fish continued to live another 24 hours, but the anemia was so acute that Cryptobia failed to survive.

At the time of this writing this study is being continued. The results thus far show that one flounder has remained infected with Cryptobia for at least 13 months, and four smaller flounders have maintained a heavy infection at least nine months without reinfection by leeches. I do not know how many additional months these flounders were infected before the start of the laboratory trials, but this data emphasizes the ease with which the infection may be perpetuated in a given area such as Great Bay. The fish remain a constant source of infection for the leeches.

Discussion

This type of longevity study has not yet been conducted with artificially infected flounders. Early trans-

mission studies showed that some flounders lost their artificial infection in eight to nine days, while other fish so infected (division studies) remained parasitized at least several weeks, when I discontinued the examinations. These longevity trials emphasize the low virulence of this species of Cryptobia in flounders, concurring with the results of the pathology trials in that these flagellates are more commensal in nature than they are parasitic. They appear to cause little or no harm to the host and the host supports their existence for a considerable period of time.

I feel these studies fail to show conclusively that multiplication of this species of Cryptobia does not occur in the vertebrate host. I was unable to demonstrate any forms of reproduction in the blood or organs of the fish, however, and my findings are largely supported by the available literature on trypanoplasms and trypanosomes. One possible area for further investigation is that of diurnal periodicity. Blood, brain, kidney and spleen smears from naturally and artificially infected flounders have been examined for reproducing forms from 6:00 AM, to the following 2:30 AM, but results were negative. Further examinations must be made at other early morning hours.

SECTION V

SPECIFICITY STUDIES

The importance of host specificity in classifying blood flagellates was pointed out by Laveran (1904) in his work with trypanosomes, and reiterated in the study of the genus Trypanoplasma. He stated,

> Les ressemblances morphologiques ne suffisent pas pour qu'on soit autorisé à identifier deux Trypanosomes ou deux Trypanoplasmas; il est démontré que certains de ces parasites, bien distincts au point de vue de l'action pathogène, se présentant à l'observateur sous des aspects à peu près identigues. J'ai donc pense qu'il serait intéressant, pour trancher la question d'unité et de dualité de ces parasites, de rechercher si le Trypanoplasme du rotengle était inoculable au vairon et réciproquement.

Speciation of the parasite was determined, then, by its host specificity as well as its morphological characteristics. Because many poorly described species of Cryptobia (Trypanoplasma) have been reported from various hosts, the host specificity of the species of Cryptobia under investigation should be determined to place it in the proper taxonomic niche.

A series of infectivity trials were organized based on the application of the method used by Laveran and Mesnil (1904). The transmission of flagellates from one fish to another by intraperitoneal injection of the organisms was effective in initiating infections of trypanosomes and trypanoplasma in freshwater fish.

Trial I

One winter flounder (11.0 cm.) and two smooth flounders (9.6 cm. and 14.0 cm.) were inoculated intraperitoneally with 0.2 cc. of an oxalate solution of concentrated Cryptobia. The organisms obtained from the blood of infected smooth and winter flounders were concentrated by the phytohemagglutination method (Yaeger, 1960). All three fish had been found negative on two successive blood examinations.

Twenty-four hours following the inoculation of the organisms, the three specimens were bled from the gills and appeared negative for the parasites. Forty-eight hours and seventy-two hours following inoculation, the winter flounder (11.0 cm.) still appeared negative, but several Cryptobia were found in the blood of the two smooth flounders. There was no apparent increase in the number of parasites observed in the positive fish, although one of the specimens appeared very sluggish and anemic. It died about one-half hour later, presumably from over-bleeding. Internal organs were removed, crushed and examined for Cryptobia, but the results were negative.

Six and eight days postinoculation, the winter flounder still appeared negative for the flagellates. The organisms in the surviving smooth flounder appeared much reduced in number. Examination eleven days after injection failed to reveal Cryptobia in either fish. The parasite had apparently disappeared from the smooth flounder. The trial

was terminated 20 days after inoculation when a further search for the flagellates was negative.

The above experiment demonstrated that it was possible to transmit Cryptobia from an infected fish to an uninfected one by intraperitoneal injection of positive blood. The trial also indicated the possibility of an immunity mechanism, whose stimulation resulted in the apparent disappearance of the flagellates from the artificially infected fish approximately eleven days following inoculation of the parasite. This result does not agree with the conclusions of the longevity studies, (page 59), where fish remained infected for several months, or with the results of the hyperinfectivity studies described on page 93 where the organisms exhibited a radical drop in number but were not completely eliminated. It must be realized, however, that the fish used in this first transmission study received but one relatively light exposure to the organism; whereas the fish in the other studies were repeatedly exposed to the parasites.

Trial II

A second trial was conducted to verify the first appearance of the organism forty-eight hours postinoculation. Upon two examinations one week apart, two smooth flounders (A=10.4 cm., B=8.8 cm.) were found negative. Both fish were inoculated via the peritoneal cavity with approximately 0.1 cc. of whole, unoxalated, infected blood from winter flounders.

Examination of the blood of these fish twenty-four hours following inoculation proved negative for Cryptobia, but the flagellates were detected in each of the fish 47 hours after injection. The few organisms that appeared at this time were long and slender in form. No sign of division was noted in these preparations.

Seven days postinoculation several organisms were seen in fresh smears from each fish. While some of the parasites now appeared broad in form, long, slender shapes still prevailed. Organisms possibly dividing were seen in the smear from fish A (10.4 cm.), and a permanent preparation was made. I found no forms of reproduction on the slide, however. Left in poorly oxygenated water for too long a time, this fish died five hours following this bleeding.

Nine days postinoculation, several non-dividing organisms were seen in the fresh preparation from fish B.

Twenty days from the time of infection, examination of the blood from fish B revealed many Cryptobia, and it seemed that multiplication within the host had occurred. No dividing organisms were seen, however. The fish was in poor condition as the gills were very pale and frayed from so much bleeding. The following day it died in the aquarium.

The results of these two transmission trials showed that passage of the flagellates from one fish to another was easily accomplished by inoculating Cryptobia positive blood into the peritoneal cavity of the susceptible host. Organisms from the winter flounder would infect the smooth flounder

and vice versa. The parasites were found in the blood of the inoculated fish two days later, a much shorter interval than the 16 to 20 day period required to detect *Trypanoplasma borreli* in the blood of the freshwater fish as reported by Laveran and Mesnil (1904). The method of transmission was used in the following host specificity trials.

Trial III

To determine the susceptibility of warm blooded animals to this species of *Cryptobia*, nine one-month old mice were used. The animals were divided into four groups and inoculated via the peritoneal cavity as follows:

Table 1

Mouse Inoculation Studies

Group	Mice inoculated	Inoculum
I (Controls)	3	0.1 cc. sterile lithium oxalate
II	2	0.1 cc. positive oxalated blood
III	2	0.1 cc. positive non-oxalated blood
IV	2	0.1 cc. concentrated *Cryptobia* suspended in saline

Two mice in the control group died fifteen minutes after the injection.

Seven days postinoculation a drop of blood from the tail of each animal was mixed with a drop of 1% oxalate and examined under the microscope. All animals appeared negative when observed for ten minutes with a 10x objective and a 20x hyperplane ocular.

Two weeks after the injection of organisms, the mice were bled again in a similar manner. No Cryptobia were observed in any of the animals in a ten minute examination of each preparation.

Another examination carried out three weeks following the inoculation likewise proved negative to the parasite. The results of this trial showed that mice were not susceptible to one inoculation of this species of Cryptobia.

Trial IV

Following this unsuccessful attempt to infect mice with Cryptobia, a multiple injection trial was conducted in an effort to break down the innate resistance which apparently existed.

Six adult mice were used in this experiment, two receiving only one dose (0.1 cc.) of positive serum during the entire trial. One of these animals died a few minutes following the injection. The other four animals received a series of six inoculations (0.1 cc. of positive serum) at daily intervals. An autopsy revealed an internal hemorrhage in the abdominal cavity of a mouse dying following the fourth injection. Two days following the last injection of the parasites, the surviving mice were examined for Cryptobia. All animals appeared negative. Seven days later another examination of the blood was likewise negative. Thus it appears, even with the repeated exposure to the organism, that mice are refractory to Cryptobia.

Trial V

Two goldfish (Carassius auratus) were inoculated via the peritoneal cavity with 0.1 cc. of unoxalated blood from an infected flounder. A drop of blood from this flounder, checked for Cryptobia at the time of the goldfish inoculation, revealed the heaviest natural infection that I had seen. There were thousands of the parasites in the one preparation, less than 0.01 ml. volume. The flounder supplying the blood appeared normal, and subsequent examinations showed a rapid decrease in the number of parasites. Three days postinoculation the blood of both goldfish was examined and appeared negative. Four hours later one of the goldfish died, but seven days following the injection the surviving fish was still negative; ten days later no Cryptobia were seen. The gills of the fish were now very pale from the repeated bleedings. The final examination of blood for this trial was made fifteen days following the inoculation. No organisms were revealed, and it was concluded that goldfish were refractory to one exposure of Cryptobia.

Trial VI

Because of the failure to infect goldfish in Trial V, a second attempt to overcome innate resistance of the host was organized. Three goldfish received multiple intraperitoneal inoculations of 0.1 cc. of serum from a positive flounder. The inoculum was checked for viable

organisms at each injection.

Three injections were given daily and on the fourth day the three goldfish were examined for Cryptobia. All specimens appeared negative. The fourth inoculation was administered at that time also, with the fifth and sixth exposures following consecutively. Twenty-four hours following the sixth inoculation, the fish were examined and still appeared negative. One week later a third survey also indicated that the goldfish were refractory to infection with the parasite.

It was concluded from Trials V and VI that there appears to be no cross infection between the species of Cryptobia commonly found in the flounders of Great Bay and the common goldfish, Carassius auratus.

Trial VII

As Walker (1910) had reported Trypanoplasma ranae from the marsh frog (Rana palustris), several specimens of frogs (Rana pipiens and R. sylvatica) from the Durham, New Hampshire area were examined for Cryptobia; however, only negative results were obtained.

I attempted to infect two frogs, (Rana pipiens) by a series of six daily intraperitoneal inoculation of 0.1 cc. positive flounder serum. No adverse effects were observed.

One of the animals, sacrificed two days following the last injection, was negative. The second frog, killed nine days postinoculation, failed to show any Cryptobia in

fresh blood smears. It appears, therefore, that the species of frog used in the trial was refractory to infection by Cryptobia found in the flounders of Great Bay. The result also tends to repudiate any synonymity of species between Trypanoplasma ranae and that of the organism under investigation.

Trial VIII

One mullet, seined at Newburyport, Massachusetts, was apparently negative to Cryptobia, even though the fish was obtained from the same pool as positive Fundulus and positive flounders. As this was the only mullet specimen caught, its negative status was not a significant criteria of its ability to harbor the parasite. Therefore a series of six daily injections of 0.1 cc. of positive flounder serum into the peritoneal cavity was given. Prior to the last inoculation the fish hemorrhaged subcutaneously with considerable loss of scales. Two hours following the last injection of serum, the fish was sacrificed and the blood examined for Cryptobia. Although the skin and scales were also examined, the flagellate was not seen in any of the several preparations. Trichodina was found in the smears from the gills, and many motile bacteria were observed in the preparations containing scales.

Trial IX

One Fundulus majalis and one F. heteroclitus, seined at Barnstable, Massachusetts, were observed as negative to

Cryptobia following two examinations. Each fish was inoculated intraperitoneally with 0.1 cc. of positive serum. Examination 48 hours following the last inoculation revealed that both Fundulus harbored the parasite. This result was not surprising as several mummichogs have been found naturally infected. The result of this trial showed the striped killifish is also susceptible to Cryptobia.

Trial X

Four brook trout, (Salvelinus fontinalis), obtained from the New Durham, New Hampshire, fish hatchery, were examined for blood flagellates. No organisms were observed; therefore each fish received 0.1 ml. of Cryptobia positive serum from infected flounders, via the peritoneal cavity. Twenty-four hours later two of the trout were dead and the two survivors were given a second inoculation of the organisms. The third dose was administered the following day.

Seven days postinoculation both trout were examined for Cryptobia, but no organisms were seen. One of the fish died following this investigation. The remaining fish was examined fourteen days following the initial inoculation, but the results remained negative for the parasites.

Trial XI

Two grubbies (M. aeneus), collected at Rye, New Hampshire, appeared negative for Cryptobia on two successive examinations. Each fish received an intraperitoneal injection of 0.1 cc. positive flounder serum. Forty-eight hours post-

inoculation no parasites were detected in either fish; a second inoculation was given. One of the sculpins (9.8 cm. long) died a few hours later, but the surviving fish (8.8 cm. long) examined three days following the second inoculation, remained negative. No Cryptobia were found 17 days later in fresh preparations. These results are interesting in view of the fact that Bullock found the parasites in a 3 1/2 inch grubby from Newcastle, New Hampshire, in 1952. I cannot explain why the sculpins from Great Bay appeared refractory.

Miscellaneous

Two tomcod, (Microgadus tomcod), caught on hand lines in Little Bay, appeared negative to Cryptobia and an attempt was made to inoculate these fish with positive flounder serum, but both fish died following the second injection. Examination of their blood at the time of death failed to reveal any Cryptobia.

A third specimen of Microgadus was caught two months later. This fish, also negative to Cryptobia, was given a series of three daily injections of positive flounder serum. Examination of its blood seven and fourteen days postinoculation failed to disclose the desired parasites. It was concluded that the tomcod was refractory to infection with the flounder species of Cryptobia.

Three yellow perch, (Perca flavescens), caught in the Durham, New Hampshire area, were negative to Cryptobia

upon examination of their blood. Because the fish were killed, attempts to artificially infect them were not possible.

Rankin (1937) reported a biflagellate described as Cryptobia borreli from the blood of North Carolina salamanders. No attempts were made to infect salamanders with the species of Cryptobia being studied, but six newts (Notophthalmus) were examined. Trypanosomes were observed in each of the animals, but no Cryptobia were found.

Discussion

Cryptobia were artificially transmitted from winter flounders to winter and smooth flounders by injecting the serum of positive fish into the peritoneal cavity of the negative specimens. Two days were required for the parasite to appear in the blood of the injected hosts after one inoculation of 0.1 cc. inoculum. Cryptobia from flounders were successfully transplanted, with one injection, to Fundulus heteroclitis and F. majalis. This transmission method was used satisfactorily in all specificity trials as well as pathology and division studies.

The possibility of an immune response to Cryptobia by the infected fish was demonstrated in these studies, particularly when multiple injections were given. A more detailed investigation of immunity is necessary but was not undertaken as a part of this dissertation.

The results of these trials demonstrated the marked inability of the flounder species of Cryptobia to be trans-

planted into laboratory mice, frogs, goldfish, brook trout, mullet, tomcod and grubbies. Multiple inoculations were given in each of these negative trials, but only one injection was necessary to produce parasites in the blood of susceptible hosts.

I consider these data, by indicating a relatively high degree of host specificity, significant in locating this species of Cryptobia taxonomically.

SECTION VI

DESCRIPTION OF THE PARASITE

Observations of live Cryptobia are readily made by examining drops of whole blood from heavily infected fish, or by concentrating the organisms in the serum, according to the technique described earlier in this paper. The live organisms in the fresh preparations are easily detected because of their quick motions. The most rapidly moving Cryptobia, the great majority of organisms, seem to remain in one spot, wriggling and writhing in patterns appearing as though tied hopelessly in knots. In these instances the flagella are discernible infrequently and only with optimum light. The parasite simply appears as a polymorphic mass of protoplasm, abounding with energy. Presumably similar activity occurs in the circulating blood of the vertebrate host. Occasionally organisms may be seen moving in a definite direction for very short distances, no longer thrashing wildly about in one spot. This directional movement is sometimes observed when the parasites leave the blood clot in whole blood preparations or in fresh brain smears. The activity of the organism is far less strenuous and possibly the somewhat slower movements of the parasite allows the flagella to assume, to some extent, a function of motility. The parasites are observed to move in the direction of the

anterior free flagellum. This action is in contrast to the movements of the trypanosomes where the flagellum attached to the undulating membrane leads the way (the trailing posterior flagellum of Cryptobia is attached to the undulating membrane). Sometimes forward motion is more pronounced in organisms that have been maintained on a glass slide for several days. In these instances many of the Cryptobia undulate slowly, possibly because the organisms are approaching death. The undulation of the membrane starts at the anterior end (near the kinetoplast) and moves posteriorly like a wave. Another wave is initiated immediately. Rapidly moving organisms do not seem to exhibit any degree of forward movement.

In fresh preparations I have seen two or more organisms attached to each other by their free flagella. They are unable to pull themselves apart and give the appearance of being firmly stuck together. In a few instances several Cryptobia have adhered to each other by their flagella, the organisms themselves radiating out from the center as spokes in a wheel. This rosette arrangement was first described in snails (Leidy 1846). Many organisms have been seen adhering to red blood cells and other pieces of debris, thus suggesting the presence of a sticky material on the flagella. The phenomenon of agglutination with trypanosomes was first described by Laveran and Mesnil in 1900; a more detailed account is given in 1901.

In determining the measurements of the flagellates,

drawings were made with the camera lucida. A 20x hyperplane ocular was used with a 97x oil immersion objective. The divisions of the micrometer scale, projected onto the drawing surface, were transferred to a piece of thin white cardboard and the 10 micra measure was then divided equally into units of one micron. The organisms were next drawn with the camera lucida at the same magnification, and measured in the manner described by Minchin (1909b). The midline of the body of the parasite was traced with a piece of thread and the thread was then measured on the scale derived from the micrometer. The flagella and other structures were measured in the same manner.

One of the most striking observations is the great inequality in size of the organisms, (Plates 1 and 2). Parasites that have been air dried, fixed in methyl alcohol and stained with Giesma ranged from 12.5 microns to 23.1 microns in length in a study involving 103 specimens from 34 fish. This size differential is apparent in parasites from the same fish, and not necessarily from one host to another. As examples, fish A 165 harbored parasites ranging from 13.0 u to 20.2 u; fish A 318 contained Cryptobia extending from 14.0 u to 21.0 u, and flagellates in fish A 333 registered between 15.9 u and 22.2 u micra long. Organisms killed by osmium tetroxide and fixed either in Schaudinn's fluid or alcohol also exhibit a comparable disparity in size, extending from 10.9 u to 21.7 micra long. Forty-three parasites from eight fish were examined to

obtain these measurements. Here also size variation appeared within one host. Fish A 342 contained Cryptobia ranging from 13.1 u to 21.7 micra long. The distribution of flagellates according to size and method of fixation is shown in Table II.

Table II

Distribution of Cryptobia by Size* and Method of Fixation**

Method of Fixation	Microns													
	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Air Dried, Methyl Alc.	-	-	1	5	9	9	14	17	13	17	10	5	1	1
Osmic Fumes Schaudinn's	1	1	1	3	7	9	4	5	5	5	3	2	-	-

*Body length, not including flagella
**A total of 103 parasites from 34 fish were measured from the alcohol-fixed group. Forty-six parasites from 8 fish were measured from the osmic-killed group.

These data point out the distribution of size from 13 to 21 micra, with both methods of fixation. Hence it is rather difficult to justify average size as valid criteria for species determination.

Laveran and Mesnil (1901) and Minchin (1909a) have indicated a great disparity in the size of trypanoplasma from the same fish. Keysselitz (1906) said the large organisms are male gametes and the small are the female

forms, but Minchin disagreed with this hypothesis. I have found nothing in my work to substantiate the contention of Keysselitz.

The following detailed measurements of the parasites were taken with both methods of fixation: (a) body length, (b) body width through the middle of the nucleus, (c) length of the anterior flagellum, (d) length of the posterior free flagellum, (e) length of kinetoplast, (f) width of kinetoplast, (g) length of the nucleus, (h) width of the nucleus, (i) the distance from the anterior end of the nucleus to the anterior end of the parasite, and (j) the distance from the anterior end of the kinetoplast to the anterior end of the animal.

The series of measurements for this species of Cryptobia, according to method of fixation, are shown in Tables II, III, and IV. The difference in mean body length and body width between the air dried methyl alcohol-Giemsa stained specimens and the osmic killed, absolute alcohol-Giemsa stained organisms (Taliaferro's method) is highly significant by the "t" test. This difference is not surprising as it upholds Minchin's contention (1909a), later substantiated by Taliaferro (1923), that greater deformation occurs in the air dried organisms than with those killed by osmic fumes. As I have never observed the parasites at the times of death, I do not know if air drying causes extension of the flagellate, or if osmic fumes causes the organisms to contract. The standard deviation (Snedecor, 1946) of

the osmic killed parasites is greater than that of the air dried organisms in all measurements but one, the length of the nucleus. This statistic shows more variation in the measurement of the flagellates killed by osmic fumes, and therefore there is more morphologic variation in the living organisms. It is important to note that a considerable range in size exists for all measurements. As I mentioned previously, the kinetoplast very nearly equals the nucleus in size, a situation quite different in trypanosomes.

The nucleus of the cell is not easily seen in the living state, but it is readily detected in stained preparations. It varies in shape from elongate to round, depending apparently on the distortion of the flagellate during fixation. The oval form appears to be normal. The nucleus lies anterior to the mid-line of the parasite and slightly posterior to the kinetoplast. Infrequently it may be seen lying adjacent to, or even slightly anterior to the kinetoplast. Usually the nucleus lies on the opposite side of the body from the kinetoplast.

When stained with Giemsa (air dried, methyl alcohol fixed) the nucleus appears rose-pink in color. No distinct karyosome is observed; instead there are scattered, irregular, dark purple granules. In some specimens these granules are large and occupy most of the nucleus, giving it a rather dense appearance.

The kinetoplast, usually rod or oblong shaped, is located anterior to the nucleus and lies an average of 1.5

microns from the extreme anterior end. It is more dense than the nucleus and stains a deep reddish-purple color. I have never observed a distinct blepharoplast in parasites stained with Giemsa. The flagella appear to arise directly from the large kinetoplast, and stain faintly pink, though in poorly stained preparations the structures are barely visible and take on a bluish tinge. The posterior flagellum, which is shorter than the anterior, borders an undulating membrane and does not always extend to the posterior end of the animal (Plate 3, 1, 2 and 6). This membrane is usually not discernible in air dried, methyl alcohol fixed specimens. Occasionally, however, this structure may be seen (Plate 3, no. 6). In a few parasites the undulating membrane may be as wide as 1.5 u, but widths of one micron or less appear common. The cytoplasm of this species of Cryptobia is alveolar, with what appear to be a few vacuoles scattered throughout. The intensity of the light blue stain varies from one area of the parasite to another, suggesting variations in thickness of the cytoplasm. Often large, darkly staining chromatin granules are seen, mostly at the posterior end below the nucleus. I have never seen myonemes in any of the flagellates stained with Giemsa (either air dried or osmic killed) or in parasites stained with iron alum hematoxylin. Leger (1905), however, has seen myonemes along the line of the insertion of the undulating membrane in Trypanoplasma intestinalis from Box boops. As Minchin (1909a) pointed out, it is necessary to use delicate

Table III

Measurements of Cryptobia sp. (Microns)
Air Dried-Methyl Alcohol Fixed-Giemsa stain (103 specimens)

	Body Length	Body Width	Anterior Flagellum	Posterior Flagellum	Length Kinetoplast	Width Kinetoplast	Length Nucleus	Width Nucleus	Anterior Nucleus to Anterior End	Anterior Kinetoplast to Anterior End
Mean	17.6	2.7	13.1	8.5	3.3	1.1	3.4	1.4	4.2	1.5
Range	12.5 to 23.1	1.2 to 4.5	8.3 to 19.1	4.4 to 15.7	1.7 to 5.5	0.6 to 2.0	1.8 to 6.8	0.6 to 3.8	0.9 to 6.3	0.1 to 4.6
Standard Deviation	2.36	0.64	2.16	1.94	0.82	0.28	0.95	0.39	0.88	0.60

Table IV

Measurements of Cryptobia sp. (Microns)
Osmic Killed-Absolute Alcohol Fix - Giemsa Stain

	Body Length	Body Width	Anterior Flagellum	Posterior Flagellum	Length Kinetoplast	Width Kinetoplast	Length Nucleus	Width Nucleus	Anterior Nucleus to Anterior End	Anterior Kinetoplast to Anterior End
Mean	16.5	4.3	13.1	8.7	3.6	1.6	3.6	1.8	4.1	1.4
Range	10.9 to 21.7	2.9 to 6.0	5.8 to 17.5	4.6 to 18.2	1.3 to 5.7	0.8 to 3.6	1.9 to 5.6	1.0 to 2.6	1.2 to 6.2	0.1 to 3.0
Standard Deviation	2.58	0.86	2.59	1.95	1.16	0.52	0.88	0.42	1.12	0.67

and exact staining procedures. Perhaps electron micrography will reveal these structures.

Organisms that have been osmic killed and fixed in absolute alcohol according to Taliaferro (1923) will stain well with Giemsa. The nuclear chromatin is rose color, with scattered dark red-blue granules. The cytoplasmic granules also stain this color. The kinetoplast shows as dark reddish-blue, and the blepharoplast fails to take the stain. The flagella are very prominent and appear rose colored. These structures are much thicker than those of the air-dried, Giemsa stained parasites. The posterior flagellum outlines the undulating membranes very clearly in most specimens. The vacuolated cytoplasm stains a rose color, variable in intensity.

When the parasites have been osmic killed, fixed in Schaudinn's and stained with iron alum hematoxylin (after Minchin (1909a), the nucleus often becomes very indistinct. Sometimes it appears as an irregular grey mass with no granules. At other times the nuclear membrane is obscured and only the dark staining granules are visible. Still no karyosome is visible. A distinct blepharoplast, unobserved with Giemsa stain, appears as a small grey to black dot, from which arise both flagella (Plate 3; 3, 4 and 5). The blepharoplast is not discernible in all parasites, due possibly to the extent of stain extraction. Minchin (1909a) found two minute blepharoplasts (in Trypanoplasma) with a fibril connecting them to the kinetoplast. I have been able to

demonstrate only one blepharoplast and no fibrils, due perhaps to inexperience with extraction techniques. The kinetoplast appears as a dense structure and stains uniformly black. The flagella show as thin filaments but are easily seen except in some instances where over-extraction occurred. The undulating membrane, bordered by the posterior flagellum, is well demonstrated with iron alum hematoxylin. The grey cytoplasm varies in intensity and sometimes contains scattered course black granules. I have never seen chromosomes in any of my preparations.

When this species of Cryptobia is osmic killed, fixed in Schaudinn's, stained with Feulgen's nuclear stain and counterstained with fast green, the morphology of the organism is indistinct. The flagella show rather clearly but the body outline is often indistinct. The dense kinetoplast stains a pink color, as does the nucleus to a somewhat lesser degree. Cytoplasmic granules or vacuoles are not observed. The organisms are very pale and optimum lighting is necessary. Phase microscopy is no improvement.

SECTION VII

PROPOSED NAME OF THE SPECIES

As indicated in the literature review, there is some question as to the correct genus in which these blood flagellates of flounders may belong, Cryptobia Leidy 1846, or Trypanoplasma Laveran and Mesnil 1901. Crawley (1909) said that morphologically the two were congeneric and even Laveran and Mesnil (1912) agreed to this, though Matthey (1923) disputed it. The majority of texts today, however, consider the two genera the same. The chief difference, then, between Cryptobia and Trypanoplasma lies in the physiological aspects of the life cycle : transmission by copulation in snails, by leeches as intermediate hosts for the blood forms, and transmission by unknown means in the intestinal forms. The main question is whether these life cycle differences justify the distinction of different genera. Crawley's reply was in the negative; Laveran and Mesnil (1912) said both genera were valid, and Matthey (1923) concurred with Laveran Mesnil. Wenyon (1926) said that all forms should actually be included in the genus Cryptobia, but the blood forms have been known so long as Trypanoplasma that the genus should be retained provisionally. Swezy (1919) said that habitat alone cannot be used as a generic distinction.

It seems to me that the key to the solution lies in the taxonomy of the intestinal forms. If life cycles are valid criteria for new genera, then we must have Cryptobia in snails, Trypanoplasma for blood forms in fish, and a new genus for the intestinal types, provided the life cycle of the intestinal forms is found to differ from that of the Gastropod or blood organisms. (Woodcock (1909) said it would be erroneous to separate the intestinal trypanoplasms into a distinct genus.) Presently the life cycle of the intestinal forms is unknown, and this lack of knowledge is perhaps the reason that this question of taxonomy has not been settled definitely over the past 60 years. If life cycles are not valid criteria for new genera, and morphology is the basis for generic distinction then I consider that Cryptobia has priority over Trypanoplasma.

The problem of speciation of the flagellates described in this dissertation is much less difficult. Morphologically I have already shown that measurements alone of the organisms cannot be considered as valid criteria for speciation since a consistently wide range occurred. Further, the measurements of the Cryptobia from flounder blood do not agree with the measurements of Trypanoplasma parmae, the only other cryptobiid found in the blood of marine fish. Physiologically, host specificity trials (considered by Laveran (1904) as valid criteria for speciation) have shown the inability of the parasite to be transplanted to several species of freshwater fish. While this perhaps does not

prove conclusively that all freshwater fish are refractory to this species of Cryptobia, it would indicate that the parasite could not maintain itself in freshwater teleosts or leeches. I have also shown the inability of the flagellate to maintain itself in the intestinal tract of its host. However, the measurements of the Cryptobia from the blood of flounders do coincide with those given by Leger (1905) for Trypanoplasma intestinalis from the intestines of Box boops, and more closely with the measurements of Trypanoplasma congeri from the stomach of Conger niger as given by Elmhirst and Martin (1910). But neither of these species live in the blood of their hosts.

Since the above described species of Cryptobia is, in my opinion, a valid species in its own right, I propose to name the parasite from the blood of the flounder Cryptobia bullocki in honor of Dr. Wilbur Bullock, the first to observe it.

Genus: CRYPTOBIA Leidy, 1846

Cryptobia bullocki, new species

Morphology: (Average body dimensions based on the measurement of 103 specimens from 34 flounders. Flagellates were air dried, methyl alcohol fixed, Giemsa stained). Body length, 17.6 (range 12.5-23.1) microns, standard deviation (s.d.) 2.36; body width 2.7 (1.2-4.5) microns, s.d. 0.64; length of anterior flagellum, 13.1 (8.3-19.1) microns, s.d. 2.16; posterior flagellum, 8.5 (4.4-15.7) microns, s.d. 1.94; length of kinetoplast, 3.3 (1.7-5.5) microns, s.d.

0.82; width kinetoplast, 1.1 (0.6-2.0) microns, s.d. 0.28; length of nucleus, 3.4 (1.8-6.8) microns, s.d. 0.95; width nucleus, 1.4 (0.6-3.8) microns, s.d. 0.39; distance from anterior edge of nucleus to anterior end of animal, 4.2 (0.9-6.3) microns, s.d. 0.88; distance from anterior edge of kinetoplast to anterior end of animal, 1.5 (0.1-4.6) microns, s.d. 0.60.

Hosts (vertebrate): Pseudopleuronectes americanus Walbaum; Liopsetta putnami Gill; Fundulus heteroclitis Linnaeus and Fundulus majalis Walbaum.

(invertebrate): marine leech, tentatively identified as Piscicola rapax Verrill, 1873 (Moore, 1898).

Type locality: Great Bay, a large tidal basin in the state of New Hampshire.

Closely Related Species: Trypanoplasma parmae Mackerras and Mackerras, 1925, from the Australian white ear (Parma microlepis), varies from 12.5 u long by 3.8 u wide to 14.7 u long by 5.0 u wide, thus being shorter and wider than Cryptobia bullocki. The anterior and posterior free flagella of Trypanoplasma parmae (18.0 to 25.0 u and 18.0 to 26.8 u respectively) are much longer in proportion to body length than the comparable structures of C. bullocki. In T. parmae the cytoplasm posterior to the trophonucleus is dense and contained numerous round or irregular chromatoid bodies, 0.25 u to 1.25 u in diameter. These bodies are much less common in C. bullocki. The measurements of Trypanoplasma (Cryptobia) borreli (Laveran and Mesnil, 1901), show

it to be slightly longer (around 20 u) and wider (3 to 4 u) than Cryptobia bullocki. The flagella of T. borreli are of equal length (15 u) whereas the similar structures of C. bullocki are unequal. T. borreli has been reported only from freshwater hosts, and specificity trials demonstrated the inability of C. bullocki to be transplanted to some freshwater and marine vertebrates.

SECTION VIII

PATHOLOGY STUDIES

The fact that some species of the genus Cryptobia (Trypanoplasma) may be pathogenic to their vertebrate hosts has been pointed out previously. Laveran (1904) indicated the ability of Trypanoplasma borreli to kill fish when the parasite was inoculated into eleven negative minnows. Nine of these fish succumbed to the infection. For some time this parasite had been under the suspicion of causing "Schlaffsucht" in carps (Hofer, 1904). Neresheimer (1911), citing the symptoms and tremendous damage of the disease in hatchery carps, considered this possible, but Plehn (1903) and Keysselitz (1906) rejected the theory. Leger (1904) stated that intense trypanoplasma infections in minnows brings about anemia. The fish refuses all nourishment and dies. Perhaps the most recent example of pathology caused by Cryptobia is that described by Wales and Wolf (1955). Organisms were found in the skin, blood, ascitic fluid (a serous fluid in the peritoneal cavity) connective tissue and the kidney of heavily infected salmon and trout. The gills of these anemic fish were pale, and the fish lethargic. There was a high mortality in yearling king salmon.

The locations of Cryptobia bullocki in the vertebrate were investigated to determine the pathogenic ability of the

organism. This inquiry was conducted on both naturally and artificially infected fish. Fresh smears and stained sections were made of various organs.

Fresh oxalated preparations revealed that 12 naturally infected flounders were heavily parasitized. These fish were critically examined to discover which organs contained Cryptobia. A piece of the organ under examination was removed from the freshly killed specimen, placed on a slide with a drop of saline and mashed with a cover slip. The results of this study are shown in the following table:

Table V

Examination for Cryptobia by Organs
Naturally Infected Fish**

	1	2	3	4	5	6	7	8	9	10	11	12
Brain	+	-	-	+	+	-	+	+	+	+	-	+
Eye	-	-	-	#	-	#	-	-	-	-	-	-
Gall bladder	-	-	-	-	-	-	-	-	-	-	-	-
Gills	#	-	-	#	-	-	-	-	-	-	-	-
Intestine	-	#	-	-	+	-	+	-	-	-	-	-
Kidney	-	-	+	-	+	+	+	+	+	+	+	+
Liver	-	-	-	-	-	-	+	+	-	-	+	+
Spleen	+	-	-	-	+	-	+	-	-	+	-	-

\# not examined
** determined by blood smear

Even though all fish were heavily infected in the blood, the penetration by the parasites into other organs was extremely

inconsistent. Generally organisms seen in any organ were few in number. Those observed in the brain often appeared thin and slender in form, whereas the Cryptobia seen in other organs presented variation in shape comparable to that of the blood forms.

Cryptobia have been found in the intestines of two flounders. These organisms, very few in number, may have resulted from blood contamination when the intestines were cut open. Leger (1905) and Elmhirst and Martin (1910) indicated that intestinal species of Trypanoplasma were extremely abundant in the alimentary canal. However, if the parasites are true inhabitants of the gut, then the question immediately arises as to the taxonomic niche these organisms must oocupy. As was indicated earlier on page 52 attempts to obtain Cryptobia in the blood after oral inoculation have thus far proved unsuccessful. If, as seems to be the case, these intestinal forms of Cryptobia demonstrate a specificity peculiar to the intestinal tract, can they be the same species as the blood forms?

Because the analysis in Table V was made on fish naturally infected, the length of time the infection had persisted in the fish is unknown. Hence, it may be possible that in the early stages of the contamination some organs may be readily parasitized by the Cryptobia. In order to check on this possibility, a second series of organ examinations was conducted on artificially infected fish which were originally negative. The results of this study are presented in

Table VI.

Table VI

Examination for *Cryptobia* by Organs Artificially Infected Fish*

Fish	1	2	3	4	5	6
Hours post-inoculation	24	24	24	24	24	24
Brain	+	+	+	-	+	-
Intestine	-	-	-	-	-	-
Kidney	+	+	-	+	-	+
Liver	-	-	-	-	-	-
Spleen	-	-	-	-	-	-
Blood	+	+	+	+	+	+

*These fish, all observed to be negative at the start of the trial, received four successive intraperitoneal inoculations of *Cryptobia* positive serum.

From the results of the foregoing experiments, the conclusion may be drawn that, even though this species of *Cryptobia* may somtimes penetrate organs via the blood stream, such migration does not seem to produce a visible pathological effect.

Another approach to the problem of pathogenicity of this parasite was an attempt to superinfect fish to the point where morbidity and mortality were manifested. Two trials to accomplish this superinfection were carried out.

The first endeavor to achieve hyperinfection involved two naturally infected *Liopsetta putnami*, 8.0 cm. and 8.1 cm. long. On August 18, 1960, both fish were

inoculated intraperitoneally with 0.1 cc. of Cryptobia positive fish serum. The second, third, and fourth inoculations, same amount and route, were administered approximately twenty-four hours apart. Thirty hours after the fourth inoculation both flounders were examined for the flagellates. The serum concentration method was not used; instead a drop of blood from the gills, collected into oxalate, was searched. The smears of whole blood from each fish revealed an extremely heavy infection of Cryptobia. The organisms were the most concentrated ever observed. The flagellates appeared almost as numerous as the blood cells.

Following the fourth injection, the flounders thus infected had appeared quite inactive for two days. The fifth, sixth, seventh and eighth intraperitoneal inoculations of 0.1 cc. infected serum were continued at daily intervals. The ninth injection was administered three days following the eighth, and the tenth injection followed the ninth by two days. The eleventh and twelfth inoculations of positive serum were given on successive days.

Twenty-four hours following the twelfth inoculation, the blood of both fish was examined for the parasites. Both flounders appeared quite thin, but they were very active.

A whole blood-oxalate smear from the first fish (8.0 cm.) revealed but two Cryptobia on the entire slide. This survey was in direct contrast to the one made earlier in which the organisms were so numerous. Autopsy of this fish which died immediately after bleeding revealed an

abdominal cavity full of blood. It is not known if this hemorrhage was the result of handling the fish at the time the blood sample was taken from the gills.

The results of the examination of the blood of the second flounder (8.1 cm.) were quite different from that of the first fish. The second flounder remained heavily infected with Cryptobia in its blood, though the organisms were much less dense than observed following the fourth inoculation.

The difference in intensity of the Cryptobia infection in these two fish, following twelve inoculations of positive serum, cannot be explained. One unknown factor which may be considered is that of the probable difference in time the fish were infected prior to the beginning of this trial. Had the first flounder (8.0 cm.) been naturally infected longer than the second (8.1 cm.) then presumably the immunity response of the first fish would have been stimulated earlier. In any event, repeated inoculation of Cryptobia failed to elicit an observable pathological response.

A second trial of superinfection was initiated in a similar manner to that of the first just described. In this instance, however, both flounders (Liopsetta, 8.8 cm. and 9.2 cm. long) were negative to three blood examinations prior to the start of the inoculations. Each fish received 0.1 cc. of Cryptobia positive serum in each intraperitoneal injection.

The first four inoculations were given to each fish

daily. The second flounder (9.2 cm.) was bled for examination seven hours after the third injection. The fresh preparation revealed a heavy infection of Cryptobia which had apparently developed in three days. No dividing forms were seen.

Three days following the fourth inoculation, the first fish was found dead in the aquarium. The cause of death was not determined. The surviving flounder received its fifth dose of Cryptobia at this time. Two days later the sixth dose was given, with the seventh and eighth inoculations following at daily intervals. Following the eighth exposure to the flagellates, the flounder was examined. Only a moderate infection was present, much less severe than was expected, based on the observations of the first of these trials.

The ninth and tenth doses were given on successive days; the eleventh dose was given three days later, followed by the twelfth inoculation in an additional two days. At this time the fish blood was examined for Cryptobia. Fewer Cryptobia were present following the twelfth exposure than were found at the time of the eighth dose of the organisms.

Discussion

The results of the pathology studies described above indicate that Cryptobia bullocki found in the fish of the Great Bay area appears relatively non-pathogenic. While no study was made of the existence of infection immunity, following constant exposure the number of parasites first

increased rather rapidly, then quite suddenly were reduced. This conclusion seems to conflict with the results of the study on the longevity of infection discussed previously. Fish harboring rather heavy infections of Cryptobia in the blood remained apparently healthy for at least eight months, the maximum time observed.

SECTION IX

DISTRIBUTION AND INCIDENCE

Great Bay, New Hampshire

Cryptobia bullocki was first observed by Bullock (1952) in flounders found in Great Bay, New Hampshire. This large tidal basin, described by Jackson (1944) in a biological survey of the area, has a shore line of approximately twenty miles and a mud bottom furnishing an excellent habitat for flounders, sculpins and killifish. Numerous freshwater streams emptying into the area produce a brackish situation with substantial variation in salinity according to location in the bay, time of tide, and rainfall.

Temperature fluctuations of the waters of the bay vary considerably more than those of the ocean. Because the water temperature is sufficiently low, ice usually covers much of Great Bay proper from January to March. Small flounders infected with the flagellates were readily obtained during this season by ice fishing in shallow areas. In warmer seasons of the year the temperature of the water averages from eight to ten degrees higher than the Atlantic Ocean off Portsmouth, New Hampshire. During this time specimens infected with *Cryptobia* were procured mostly by beach seining on the flats or in the small streams emptying into the bay.

Because of the proximity of Great Bay to the campus of the University of New Hampshire, this region provided the greatest numbers of fish for study. Specimens were collected mainly from four areas of the bay. Because of its easy accessibility Crommet Creek contributed the greatest number of flounders. Many fish were taken from Little Bay, adjoining Great Bay, but these were larger specimens obtained primarily for leeches that may have been attached to them. Specimens were also taken at Emerson's Beach and in Greenland Bay in the vicinity of Fabyan's Point, Newington.

A total of 271 fish were taken from Great Bay from September, 1959, to March, 1961. The following table shows the species of fish obtained and the number of specimens containing *Cryptobia* in their blood.

Table VII

Incidence of *Cryptobia* Infection in Great Bay, N. H.

Species	Fish Positive	Fish Negative
Smooth flounder (*L. putnami*)	113 (+9)*	44 (5)
Winter flounder (*P. americanus*)	60 (+19)	26 (10)
Grubby (*M. aeneus*)	(1)	9
Mummichog (*F. heteroclitis*)	1	6
Striped killifish (*F. majalis*)	1	6
American Eel (*A. rostrata*)	0	3
Atlantic Tomcod (*M. tomcod*)	0	2

* Bullock's data

The above data shows that both species of flounders

are commonly parasitized by the flagellates in the Great Bay area. Sixty-eight per cent of the smooth flounders and 55% of the winter flounders were infected. The incidence of parasitism in both species of Fundulus and the grubby was considerably lower, though fewer fish were examined.

The salinity, measured at low tide, was 27 parts per thousand at Crommet Creek and Fabyan's Point. These recordings were made in the middle of August during a period of relatively low rainfall. Salinity as low as 24 parts per thousand has been reported by Jackson (1944), however. The normal salinity of the open sea ranges from 34 to 36 parts per thousand.

An unidentified trypanosome was found, on two different occasions, in the blood of Liopsetta putnami from Crommet Creek, Great Bay, New Hampshire.

Portsmouth Harbor, New Hampshire

Fish were taken from three locations in the Portsmouth Harbor area. This region is the mouth of the Piscataqua River and is where Great Bay opens into the Atlantic Ocean. In the harbor itself, on the Kittery, Maine, side of the river, five specimens of Pseudopleuronectes americanus were caught on hand lines during July, 1960. The fish ranged in size from 21.4 cm. to 23.5 cm. long and all appeared negative for Cryptobia. As indicated in Table VIII page 111 the larger fish presented an extremely low incidence of infection of the parasite; therefore, it is not surprising that these specimens appeared negative.

A second area of Portsmouth Harbor providing flounders is encircled by the following islands: Marvin, Goat, Newcastle, Fest, Leach and Salters. The salinity of the water at low tide was 31 parts per thousand, nearly comparable to that of the open sea. Ten fish were caught on hand lines in this relatively shallow body of water having depths to 19 feet at low tide. Nine winter flounders (P. americanus) ranged in size from 20.0 cm. to 27.4 cm. long, and one longhorn sculpin (M. octodecimspinosus) was 17.5 cm. long. Two of the flounders (26.0 cm. and 23.0 cm.) had Cryptobia in their blood, but the sculpin appeared negative. Attempts to obtain flounders by beach seining were unsuccessful at this location.

The third section of the harbor yielding specimens was north of, and adjacent to, the bridge connecting Newcastle with Portsmouth. This site, opposite Blunts Island, readily lends itself to beach seining. All fish caught were obtained in this manner. A muddy bottom gave way to fine sand as the beach was approached. The salinity of the water at low tide was 31 parts per thousand.

The first group of fish from this location examined for the flagellates was taken in October, 1959. Four winter flounders and two smooth flounders, ranging in size from 5 cm. to 8.5 cm. long, appeared negative. The following May, 1960, twelve more specimens, seven winter flounders and five smooth flounders, ranging from 6.7 cm. to 15.2 cm. long, were examined for the blood parasites. Again all appeared

negative. In June, 1960, two of five smooth flounders and two of four winter flounders were found positive for the organisms. Six more flounders were seined in July, 1960, one specimen containing Cryptobia in its blood.

This data suggests the possibility of seasonal distribution of the parasite in this location. A total of 18 specimens, optimum in size for infection, appeared negative to Cryptobia in October and early May. Seven weeks later in June the parasites were detected in four flounders. No leeches were found on any of the specimens. A more detailed survey is necessary before this question of seasonal distribution can be answered.

Hampton Harbor, New Hampshire

When it had been determined that a heavy incidence of infection of Cryptobia bullocki occurred in the flounders of Great Bay, and to a considerably lesser extent in the waters of Portsmouth Harbor, investigations were carried out on fish obtained north and south of these areas. Hampton Harbor was selected because flounders were known to be relatively abundant in the area.

In July, 1960, 19 fish were obtained by beach seining in a sandy area, located approximately 1000 yards north of Locke Point at the mouth of a small stream entering Hampton Harbor. Of 15 smooth flounders (7.9 cm. to 12.5 cm.), seven were positive for Cryptobia. Three winter flounders (7.0 cm. to 8.4 cm.) yielded one positive specimen. It is noted that all flounders caught were optimum in size for infection.

One grubby (*M. aeneus*) was negative. No attempt was made to study the incidence of infection, but it is evident that the organism is a common parasite of the area. A salinity of 31 parts per thousand was higher than expected, due to the large quantity of fresh water deposited into the harbor by the Hampton River. Tide water from the ocean, however, fills the entire harbor - hence the salinity.

Newburyport, Massachusetts

An attempt was made to obtain fish from some small streams entering the mouth of the Merrimack River. Hauls were made in a tributary of Plum Island River, near Seal Island in the Jopper Flats region by using a ten foot common sense seine. The location was, in some respects, very similar to Crommet Creek in Great Bay, New Hampshire. The stream was narrow, about four feet deep at low tide, and had a very muddy bottom. *Fundulus* were extremely numerous and flounders much less so, though native fishermen reported that many flounders were frequently caught there.

On August 12, 1960, the following fish were examined: 5 smooth flounders (11.3 cm. to 14.2 cm.), 2 being positive for *Cryptobia*; two winter flounders (11.6 cm. to 15.6 cm.), neither appearing to contain the flagellates; two specimens of the common alewife (*Alosa pseudoharengus*) were apparently negative, as was one mullet (*Mugil cephalus*). Four specimens of *Fundulus majalis* appeared negative. One of four *F. heteroclitus* contained *Cryptobia*, as well as an unidentified species of trypanosome. The infection of *Cryptobia* in all

positive specimens was relatively light. The salinity of the water from which these fish were taken was 24 parts per thousand.

Duxbury, Massachusetts

Three winter flounders were seined at the town beach near the mouth of the Back River in Duxbury in June, 1960. The fish (7.4 cm. to 10.2 cm. long) were all apparently negative for the blood parasites.

Again in September, 1960, the sandy bottom of the same area produced four more winter flounders, none of which appeared to harbor Cryptobia. The size of these specimens ranged from 5.3 cm. to 12.0 cm. No recording was made of salinity.

Attempts to acquire more fish from this location at this time were rendered impossible by approaching Hurricane Donna. Gusts of wind made seining with the thirty foot net practically hopeless. Abnormally high tides due to the storm perhaps also influenced adversely the results of the collecting trip.

Sippiwisset, Massachusetts

In September, 1960, an attempt was made to procure specimens for Cryptobia examination from the south side of Cape Cod in Buzzards Bay. The waters of this region were considerably warmer than those from any location examined thus far. The area fished was the town beach, Sippiwisset, at the mouth of the Sippiwisset Creek. The collecting was

done the evening before the advent of Hurricane Donna. The author remembers this as one of the most pleasant collecting trips undertaken during the course of these studies. The sea was calm, the water temperature characteristically warm and the bottom of the seining area was hard packed sand. There were no insects to irritate the collectors.

Several seine hauls across the creek and the surrounding area produced many Fundulus, but no flounders were obtained. Only five F. heteroclitus and three F. majalis were examined. All fish appeared negative for the parasites, as did two mullet (M. cephalus). No record was made of the salinity, but it would presumably be comparable to that of the open sea.

A trypanosome was seen in one F. heteroclitus, and an organism somewhat resembling a trypanosome was observed in one F. majalis, except that no flagellum was seen. The undulating movements were very slow, but the morphology was difficult to determine as these were fresh preparations. No other organisms were found by examination of Giemsa stained smears.

Barnstable, Massachusetts

Collecting at this location on the north side of Cape Cod Bay was done in September, 1960, the morning that Hurricane Donna approached Cape Cod. The water was considerably cooler here than at Sippiwisset. High winds, increasing in intensity, and a higher than normal incoming tide necessitated reducing the time allowed for gathering specimens.

Several short hauls with the large seine produced numerous F. majalis and F. heteroclitus, but no flounders were obtained. Eight striped killifish and six mummichogs appeared negative for Cryptobia when examined in the usual manner. No record was made of salinity.

York, Maine

Even though the flounders were reported plentiful in the waters of York Harbor and York River, Maine, two attempts were necessary to obtain specimens for examination. The first effort was made in June, 1960, by seining at low tide with the thirty foot net along the sand and mud flats of the York River, near its entrance into York Harbor. Several Fundulus and some sticklebacks were obtained but no flounders were found. It seemed quite likely, however, that future seining efforts might yield the desired flatfish, as the characteristics of the area appeared propitious for their habitation.

The second endeavor was made in July, 1960, by hand line fishing from the bridge crossing York River on route 103. The fish caught by local sportsmen were bled when landed, and the blood serum was examined for Cryptobia later in the day at the laboratory. Eight winter flounders (P. americanus) were obtained and examined, as well as one longhorn sculpin and one skate. Seven of the flounders were rather large, ranging from 13.6 cm. to 28.0 cm. long. None of these fish appeared infected with the flagellate. The eighth flounder, however, the smallest of those caught

(9.2 cm.) was positive for the parasite. Neither the skate nor the sculpin appeared to harbor the organisms. No salinity determination was made.

It is interesting to note that of the ten fish examined, only one was positive for Cryptobia. I feel that such an incidence is probably not indicative of the positive parasite population in the smaller, younger flounders.

Kennebunkport, Maine

In July, 1960, an effort was made to obtain fish for blood examination by seining the sandy stretches of the Mousam River near route 9 at low tide. Several seine hauls netted many Fundulus but no flounders. Two large winter flounders, however, were picked up from the sand at the water's edge. The large one (25.0 cm.) appeared ill and was easily retrieved. The fish died shortly after it was bled from the gills. The other flounder was already dead when found, yet the blood had not coagulated so a sample was obtained. Externally both fish showed damage from hemorrhage in the area of the ventral anal fin. The cause was unknown. Neither fish appeared to harbor any blood parasites. Three of 12 Fundulus heteroclitus were positive for Cryptobia. No determination of salinity was made.

Harpswell, Maine

Three large winter flounders (P. americanus), ranging in size from 22.7 cm. to 25.4 cm. long, were obtained by hook and line fishing from the waters of Harpswell Sound in

June, 1959. Three more of these flounders (21.2 cm. to 27.6 cm. long) were acquired by the same method in August, 1959. All six fish appeared negative for Cryptobia.

In August, 1960, another attempt was made to procure smaller flounders by beach seining. Several hauls with the long net yielded two winter flounders (18.3 and 14.5 cm. long), two small flounders (5.2 cm. and 4.9 cm. long), and two Fundulus heteroclitus. All these fish were examined and appeared negative to blood flagellates. A salinity determination of the water taken from the seining area was 31 parts per thousand. A small brook emptying into the mud bottom area served, at low tide, to reduce the salinity below that found in the sound.

Boothbay Harbor, Maine

In the summer of 1959 when the field work dealing with the marine forms of Cryptobia was initiated, I had an opportunity to examine the fish held in the public display aquaria of the U. S. Dept. of Fisheries Laboratory, Boothbay Harbor, Maine. The specimens had been obtained from the Boothbay region. In the following account the adult size suggests immediately that if infection were present, the incidence would very likely be low. The fish examined, with their size range, are as follows:

Winter flounder (P. americanus)	(23.5 cm.-29.8 cm.)	12 fish
Shorthorn sculpin (Myoxocephalus scorpius)	(21.7 cm.-34.5 cm.)	6
Longhorn sculpin (M. octodecimspinosis)	(22.1 cm.-27.5 cm.)	5

Ocean pout (*Macrozoarces americanus*)	(41 cm.-43 cm.)	2 fish
Sea raven (*Hemitripterus americanus*)	(35.6 cm.-43.2 cm.)	3
Cod (*Gadus callarias*)	(31.4 cm.-35. cm.)	5

Cryptobia were not observed in any of these specimens.

Rockport, Maine

Twenty winter flounders (*P. americanus*) were obtained in August, 1960, by beach seining at Glen Cove, near Rockport, Maine. Of all the areas fished during these studies, this locality provided the easiest and most fruitful seining. One haul over the hard packed sandy bottom at low tide yielded an ample number of specimens. The range in size of fish was from 6.3 cm. to 12.0 cm. long, optimum for the occurrence of *Cryptobia*.

All 20 of the flounders appeared negative for the blood parasites, as did two tomcod. The waters of Glen Cove were definitely not brackish nature. No fresh water streams of any size emptied their contents into the cove.

Otter Creek, Mount Desert, Maine

The eastern-most locality examined for the presence of *Cryptobia* was at Otter Creek on Mt. Desert Island. The seining - rather difficult because of the extremely soft, muddy bottom and excess debris such as sticks, stumps, cans, etc. - was performed in a large tidal pool where Otter Creek emptied into Otter Cove. Ocean water was exchanged during

tide changes by three large culverts passing under the pavement. The first two or three hauls were best, yielding five winter flounders and five sculpins. The size of the flounders was from 4.5 cm. to 16.6 cm., and that of the sculpins from 5.4 cm. to 9.8 cm., an optimum range for the presence of the parasite. All ten fish appeared negative for Cryptobia. No salinity determination was made of this water at low tide.

Discussion

Incidence of Infection. A compilation of the data from the Cryptobia-positive locations - Great Bay, Portsmouth Harbor, New Hampshire; York, Maine; and Newburyport, Massachusetts - reveals the relationship between size, incidence, species and age of the flounders.

The relationship of the size of the fish to the incidence of infection is observed in Table VIII. The larger fish, ranging in size from 15 cm. to 30 cm. long (approximately six to twelve inches), were 14% positive for Cryptobia; whereas the group with the greatest number of specimens (7.0 cm. to 9.9 cm.) was 77% positive. While no attempts were made to demonstrate quantitatively the intensity of infection in the fish, the parasites found in a specific time limit were much fewer in number in the larger specimens than in the smaller ones. The size range from 6 cm. to 12 cm. appeared optimum for incidence and severity of infection.

The above data shows no indication of a seasonal incidence of Cryptobia bullocki in the blood of the flounder

Table VIII

Relationship of Infection to Size of Fish and Season of Year (Smooth and Winter Flounders)*

Length in cm.	January		February		March		April		May		June		July		August		September		October		November		December	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
4-4.9																1								
5-5.9														2	1	1		4		1				
6-6.9										2	2	1	1	1			1	1		1				
7-7.9				1						5	19	1	3	2	2	3	4	3		6				
8-8.9	1		1	1	1					2	8		10	3	32	5	17			2	1			
9-9.9	1		3	2	1						2		1	2	16	5	9		1		1	1		
10-10.9			7		3	3				1	2	1	3	6	7	1	3	2	4		1			
11-11.9			7	5	1	4				1	1	1	2	2	2	1	2		1	1	1			
12-12.9			4	3		2					3	1		3	2	3	4	2	4					
13-13.9			1	1										1	3	1	1	1	1					
14-14.9											1					4	1	1	1					
15-16.9			1	1		1				1		2		1	1	1		1						
17-19.9												2		2						1				
20-24.9													1	15										
25-30													1	5										
Totals	2	2	24	14	6	10	0	0	0	12	39	9	22	45	66	26	43	15	12	12	4	1	0	0

All Little Harbor

No fish examined

* Includes 43 flounders recorded by Bullock in 1953.

species examined. No specimens were obtained in either December or April due to the lack of sufficient ice cover in the bay. Attempts at beach seining were unsuccessful during this time. No positive flounders were obtained during the month of May, but all specimens examined were from Little Harbor. Unfortunately no fish were obtained from the Great Bay area. However, it is quite unlikely that flounders would lose their infection during the months of April and May, especially in view of the fact that laboratory trials (see longevity of infection, p. 59) showed that flounders remain infected several months, even when isolated from the intermediate host.

Lack of seasonal incidence of the parasite under investigation is in contrast to the distribution of Cryptobia described by Davison, et al (1954) as C. salmositica from the silver salmon was not found during the summer months.

The data in Table IX show that the incidence of Cryptobia infection is higher in smooth flounders (70% positive) than in winter flounders (48% positive). This result is not surprising as it has been determined from Table VIII that the greatest number of positive specimens ranged from 7.0 cm. to 9.9 cm. long. Only two smooth flounders of 181 specimens exceeded 15 cm. in length, whereas 30 of 81 winter flounders were in this category. The incidence of infection in winter flounders increases to 60% positive in fish 6.0 cm. to 10.9 cm. long, and 76% positive

Table IX

Relationship of Infection to Size and Species of Flounder*

	Winter Flounders (*P. americanus*)		Smooth Flounders (*L. putnami*)	
Length (cm.)	Positive	Negative	Positive	Negative
4- 4.9		1		
5- 5.9		3		5
6- 6.9	3	3	1	3
7- 7.9	14	12	14	7
8- 8.9	30	6	41	5
9- 9.9	6	2	30	7
10-10.9	5	3	16	12
11-11.9	4	4	7	10
12-12.9	2	5	10	8
13-13.9		1	5	3
14-14.9		1	2	5
15-16.9		5	1	2
17-19.9		3		
20-24.9	2	15		
25-30	1	4		
Totals	67	68	127	67

*To March 31, 1961

for smooth flounders included in this size range.

A comparison of the relationship of size and incidence of infection to the age of flounders shows that most winter flounders become infected during the first year of life when, according to Bigelow and Schroeder (1953), winter flounders may attain lengths up to 15 cm. Little is known of the life of smooth flounders, but scales of specimens 9.6 cm. long revealed that the fish was less than one year old.

Geographical Distribution. Fish from several areas along the northern New England coast were examined for Cryptobia. The results of this survey, presented in Table X, show that the parasite is common in small flounders from some of the colder brackish water areas of the coasts of Maine, New Hampshire, and northern Massachusetts. Fish positive for the organisms were obtained at Newburyport, Massachusetts; Hampton Harbor, Portsmouth Harbor, Great Bay, New Hampshire; York and Kennebunkport, Maine. Both flounders and Fundulus were susceptible to infection by the parasite. While infected fish have been found in waters with a salinity approaching that of the open sea, a brackish water environment seems favorable.

To date the organism has not been found in the waters of the Cape Cod region (Barnstable and Sippiwisset) nor at Duxbury and Ipswich, Massachusetts. Fish negative for Cryptobia have also been examined at the following locations in Maine: Harpswell, Boothbay Harbor, Rockport

(Glen Cove), and Otter Creek, Mt. Desert. I am not satisfied with the negative results from these areas. The findings at Barnstable, Sippiwisset, and Ipswich, Massachusetts, were based entirely on the inability of finding Cryptobia in Fundulus. As no flounders were collected, these species must be obtained and examined.

Table X

Summary of Distribution and Incidence of Cryptobia from Marine Fishes of Northern New England

Area	Total Number Fish Examined	Number Positive	Average Salinity at low tide
Great Bay	253	167	27
Portsmouth Harbor	54	7	31
Harpswell, Maine	12	0	31
Boothbay Harbor, Maine*	33	0	open sea
Duxbury, Mass.	7	0	unknown
Hampton Harbor	19	8	31
York, Maine	10	1	brackish
Kennebunkport, Maine	12	3	brackish
Rockport, Maine	20	0	open sea
Mt. Desert, Maine	10	0	brackish
Newburyport, Mass.	18	3	24
Ipswich, Mass.	16	0	31
Sippiwisset, Mass.	10	0	unknown

*from aquaria tanks, not from harbor

Too few fish for valid negative results were collected at Duxbury, Massachusetts and Otter Creek, Mt. Desert, Maine. Likewise, fish larger than the optimum size for infection were examined from Harpswell and Boothbay Harbor, Maine. While the role of salinity has perhaps not been definitely established, the specimens from Harpswell, Boothbay Harbor, and Rockport, Maine, were collected in waters having salt concentrations equivalent to the open ocean. Before a region can be declared free of Cryptobia a large number of flounders, optimum in size, must be examined from brackish water habitats.

SECTION X

CULTURE ATTEMPTS

When these trials were begun, I was interested in the *in vitro* culture of the flagellates as a method of diagnosis, a procedure used by Novy and MacNeal (1905) to detect trypanosomes of birds. But as techniques for more rapid diagnosis developed (page 28), I discarded culture trials.

There are many reports in the literature of successful culture of trypanosomes from freshwater vertebrates, and these data served as a basis for my limited efforts. Ponselle (1913a, 1913b) described a media that supported *Trypanoplasma varium* Leger from the blood of *Cobitis barbatula*. While I was able to maintain *Cryptobia bullocki* for three to four days in Ponselle's media, I did not observe reproduction and the culture was lost. In 1924 Ponselle published the techniques and media for successful culture of pathogenic trypanosomes. He added inactivated rabbit serum to a solution of protein, gelatin and salts. This media failed to support growth of the flagellate from flounder blood. A change from rabbit serum to frog serum was no better, but flounder serum from *Cryptobia* negative fish would maintain *C. bullocki* for four days. Novy and MacNeal's N-N-N- media (1905) was also inadequate. I was

able to support the parasites for five days by adding several drops of infected whole flounder blood to a sodium chloride concentration of 0.8%, but multiplication of the organisms did not occur. As I reported on page 58, I was able to maintain C. bullocki for nearly two weeks using Robertson's (1911) method, but no reproduction of the parasites resulted.

It seems likely that Cryptobia bullocki from the blood of marine fish may have slightly different requirements for growth and reproduction than those of flagellates from freshwater teleosts. Thus far there are no reports in the literature that are concerned with the culture of flagellates from the blood of marine fishes. I intend to continue these studies in the future.

SECTION XI

GENERAL DISCUSSION

One of the most frustrating aspects of the study were those trials involving the life cycle of Cryptobia bullocki as it occurs in the leech. The literature of Brumpt (1906b) and Robertson (1911) was deceptive in the ease with which they carried out their investigations. Both workers had large numbers of leeches available. It is my opinion that the leeches involved in the life cycle of Cryptobia bullocki are more plentiful in nature than they have appeared, but the annelids are difficult to obtain because they readily release themselves when their hosts are disturbed. The majority of flounders caught had no leeches attached to them, and I have rarely found more than two leeches on any one fish. This situation is quite different from that indicated by Katz, who has found 90 or more leeches on one adult salmon.

The leeches I used in the laboratory trials had the ability to live a considerable period of time without feeding, a fact that was quite unknown to me when I started these trials. With the knowledge now available I am continuing the study of the life cycle of C. bullocki in the invertebrate host. Although I have not observed reproduction of C. bullocki in the leech, it appears thus far that the

majority, if not all, multiplication occurs in the annelids. I am confident that this question can be answered by obtaining many negative leeches over a period of time, holding them in isolation at least three months to develop a need for food, and then placing them in as natural an environment as possible. It may be necessary to attempt rearing leeches in the laboratory. Also, this would enable me to establish if newly hatched leeches are already infected with Cryptobia from their parents. As the majority of leeches I have examined have been negative, this is perhaps questionable. Though I have demonstrated that C. bullocki appears to be relatively nonpathogenic for flounders, I do not know the effect of the flagellates on the leech. Neresheimer (1911) stated that in cases of intense infections with Cryptobia, the leeches enlarge, discolor and die. Another unanswered question pertains to size and age factors involved in the susceptibility of leeches to the flagellate. Are the larger leeches more refractory to infection?

Notwithstanding the lack of knowledge relative to reproduction of Cryptobia in the annelid, the life cycle of the flagellate continues when the leech feeds on the flounder. At this time the Cryptobia are released from the leech and injected into the fish, probably into the lymph as I have never seen erythrocytes in any of the leeches I have removed from flounders. (This is in sharp contrast to the red blood cells I found in a leech taken from a pickerel.) After injection into the fish, the organisms are carried by the lymph

to the blood and hence to various organs such as brain and kidney where the flagellates are frequently found. I have not demonstrated to my satisfaction that multiplication of the flagellates occurs in the vertebrate host. My results are substantiated by Plehn (1903), Minchin (1909b) and Robertson (1911), none of whom observed reproduction of the parasites in the blood of fish. This incompletely investigated process of development suggests that the leech is the definitive host and the fish serves as an intermediate or accidental host.

The data collected in this study show that young flounders, mostly yearlings, may become infected with Cryptobia bullocki and remain so for many months, thus serving as a constant source of reinfection for more leeches. Seventy-seven per cent of the flounders from Great Bay, ranging in length from 7.0 cm. to 9.9 cm., were infected with Cryptobia bullocki, whereas the larger fish from 15 cm. to 30 cm. long (6 to 12 inches) were only 14% positive. It should be mentioned here that as the parasite incidence decreases the diagnostic error increases, and fish recorded as negative may actually harbor a few undetected flagellates. According to Bigelow and Schroeder (1953) the winter flounder, P. americanus, is hatched in the spring and may grow from four to six inches at one year of age (10 to 15 cm. long). This species of flounder may reach five to seven and one-half inches long at two years (18.5 cm.), and be nine or ten inches long at four years of age (23 to 26 cm.).

Similar information for the smooth flounder, L. putnami, is lacking, but young fish may reach three to four inches (7.5 to 10 cm.) the first year. Most heavily infected flounders become parasitized the first year and seem to be more resistant to infection with increasing age. One of the larger aquarium flounders (15 cm.) has remained infected with Cryptobia at a very low level of infection for over a year. I have observed neither an increase nor decrease in the number of flagellates in fresh smears, and the infection appears to remain static.

The problem of immunity to C. bullocki is an interesting one and requires further study, as the data collected presents conflicting results. As reported on page 96 of the pathology studies, a marked reduction in the number of flagellates in the blood occurred when the fish were artificially infected by a series of inoculations with the parasites. There were fewer organisms following 12 injections than there were following four to eight exposures. (The inoculum used for these injections consisted of serum from positive fish; thus I was passively immunizing the flounders). The longevity trials, approximating what occurs in nature, tend to reject the theory that the fish are protected by active immunization as no decrease in the number of parasites occurred after several months of infection. The antibody mechanism may be one of infection immunity, where antibody occurs as long as the parasite is present. This situation emphasizes the need for basic studies in host-parasite relationships.

Why is Cryptobia bullocki relatively nonpathogenic for flounders, while another species of Cryptobia is capable of producing disease in young trout and salmon (Wales and Wolf, 1955)? I hope to enlarge the investigation of this question in the future. Such work will require the in vitro culture of C. bullocki for antigen purposes, a project that has not been attempted to any extent as yet.

The geographic distribution studies show that the organism is much more common than originally believed, but the investigation has not answered the question of what factors limit the distribution of the parasite. Moore (1898) reported finding the leech Piscicola rapax on Pleuronectes dentatus in the Woods Hole region of Cape Cod, but there is still some question if the leech involved in these studies is P. rapax. The matter of leech specificity for the flagellate requires further work also. How many species of Hirudinea can transmit the parasite? The role of salinity may also be important to the leech and to Cryptobia, as the heaviest concentration of C. bullocki appear to occur in brackish water situations. Will the leech live in the salinity of the open sea? What is the ecological range of the leech? The influence of water temperature on parasitism is also unknown. As opportunity permits, further collecting along the New England Coast of optimum size flounders will be conducted with a view towards answering some of the above questions.

One other point of interest that should be mentioned

here is the fact that the kinetoplast of Cryptobia bullocki, as well as the nucleus, stains pink with Feulgen's nuclear reaction. This histochemical procedure designated the presence of DNA, a substance thus far found only in the nucleus. This result indicates that these flagellates may have two nuclei, thus substantiating Hartmann and Jollos (1910) for erecting the order Binucleata as a distinct taxonomic group for the hemoflagellates.

SECTION XII

SUMMARY OF PRINCIPLE RESULTS

1. Cryptobia bullocki n. sp. is described from the blood of some marine fishes found in Great Bay, New Hampshire. Host records and morphological characters indicate that this is a new species.
2. The hemoflagellate is assigned to the genus Cryptobia Leidy because of the priority of this genus to Trypanoplasma Laveran and Mesnil. Lack of knowledge on the life cycle of intestinal forms of the parasite prohibits separating blood, intestinal, and Gastropod forms into different genera.
3. The intermediate host of Cryptobia bullocki appears to be a leech provisionally designated as Piscicola rapax Verrill 1873, (Moore 1899).
4. Attempts were unsuccessful to demonstrate C. bullocki in leeches from rock gunnels and sculpins.
5. Argulus does not appear capable of transmitting the flagellate.
6. Reproducing forms of Cryptobia bullocki were not observed in the vertebrate hosts.
7. There was no indication of active immunization against C. bullocki. One flounder maintained the parasite 13 months, and other flounders have remained infected eight

months, the period of observation to date.

8. Cryptobia bullocki was found in the blood of the following hosts: winter flounder (P. americanus); smooth flounder (L. putnami); mummichog (Fundulus heteroclitus); striped killifish (F. majalis); and in one grubby (M. aeneus. The parasites were also found in the brain, kidney, and spleen of infected fish. A few organisms were found in intestinal smears, but conclusive evidence is lacking that these parasites are normal intestinal inhabitants.

9. Attempts were unsuccessful to artificially transmit Cryptobia bullocki to the following: mice (Mus musculus); goldfish (Carassius auratus); frogs (Rana pipiens); mullet (Mugil cephalus); brook trout (Salvelinus fontinalis); tomcod (Microgadus tomcod); and the grubby (M. aeneus).

10. Attempts were unsuccessful to transmit Cryptobia bullocki to the blood of negative fish by oral administration of the flagellates.

11. Cryptobia bullocki appears to be relatively nonpathogenic for winter and smooth flounders. Attempts were unsuccessful to produce pathology by multiple inoculations of the parasite into negative hosts.

12. Cryptobia bullocki was found in the blood of marine fishes from the following locations: Great Bay, New Hampshire; Portsmouth Harbor, New Hampshire; Hampton Harbor, New Hampshire; Newburyport, Massachusetts; York, Maine; and

Kennebunkport, Maine.

13. Cryptobia bullocki was not found in fish south of Newburyport, Massachusetts to Cape Cod, or north of Kennebunkport, Maine to Mr. Desert.

14. Seventy-seven per cent of the flounders from 7.0 to 9.9 cm. were infected with C. bullocki (probably yearlings), whereas flounders 15 cm. to 30 cm. long were only 14% infected.

15. There was no indication of seasonal distribution of C. bullocki.

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APPENDIX I

FORMULARY OF STAINS AND PROCEDURES

Giemsa Stain

Stock Solution:

Giemsa powder	0.5 gm.
Glycerin (dissolve powder in glycerin 1-2 hours at 60° C.	33.0 cc
Methyl alcohol, absolute (acetone free)	33.0 cc

Stain:

One cc of stock solution

Two cc of pH 6.5 buffer (317 ml of M/10 Na_2HPO_4 plus 683 ml M/10 NaH_2PO_4)

47 cc distilled water

Procedure:

1. Blood smears are air dried.
2. Films are fixed in methyl alcohol five to ten minutes.
3. Films are stained 60 to 120 minutes.
4. Films are rinsed in buffered tap water and then dried.

Taliaferro's Method of Staining (Taliaferro, 1923)

Stain: Giemsa as prepared above.

Procedure:

1. Wet blood smears are exposed to 2% osmic acid vapor for 30 to 60 seconds.
2. Smears are fixed in absolute alcohol five to ten minutes while still wet.
3. 95% alcohol, 5 - 10 minutes.

4. 80% alcohol, 5 - 10 minutes.
5. Bleached in 95% alcohol (40 cc alcohol and 10 cc of 30% H_2O_2.)
6. Dried in air.
7. Stained with Giemsa for 3½ hours.
8. Washed in distilled water.
9. Dried in air, mounted in balsam.

Heidenhain's-Alum Hematoxylin (after Minchin, 1909)

Procedure:

1. Wet smear in 4% osmic acid fumes, 30 to 60 seconds.
2. Wet smears are fixed in Schaudinn's fluid at 60° C for 10 minutes.
3. 3½% iron-alum, overnight.
4. Wash briefly in distilled water.
5. Aqueous hematoxylin, 0.5%, 24 hours or more.
6. Destain in iron-alum until the color is seen coming out. Tap water stops extraction.
7. Dehydrate in alcohols, 2 minutes each.
8. Clear in 2 changes of xylol.
9. Mount in balsam.

Feulgen's Nuclear Reaction

Procedure:

1.	Wet smears fixed in Schaudinn's at 60° C.	10 minutes
2.	95% alcohol and iodine	5 minutes
3.	80% alcohol	5 minutes
4.	70% alcohol	5 minutes
5.	50% alcohol	5 minutes
6.	35% alcohol	5 minutes

7.	10% Perchloric acid	12-24 hours
8.	Schiff's Reagent	20-30 minutes
9.	Wash in three, 2 minute changes of $NaHSO_3$	
10.	Wash in running water	5-10 minutes
11.	Dehydrate - 35%- 50%- 85% alcohol	2 minutes each
12.	Fast green	10-15 minutes
13.	Absolute alcohol	2-3 minutes
14.	Absolute alcohol: xylol, (50:50)	2-3 minutes

Leech Whole Mounts

Procedure:

1.	Relax animal in MS 222	
2.	Fix in Bouin's solution overnight or	
	Fix in Demke's (AFA) solution 24 hours or longer	
3.	70% alcohol	1-2 hours or overnight
4.	85% alcohol	1 hour
5.	95% alcohol	1 hour
6.	Absolute alcohol	1 hour
7.	25% Turpineol, 75% absolute alcohol-	12-24 hours
8.	50% Turpineol, 50% absolute alcohol-	12-24 hours
9.	75% Turpineol, 25% absolute alcohol-	12-24 hours
10.	100% Turpineol- 1-3 days	
11.	Mount in balsam.	

Leech Serial Sections

In preparing leeches for serial sections, the procedures of relaxation with MS 222, fixation with either Bouin's or Demke's fluids, and dehydration were the same as

those used in the whole mount preparations. Following dehydration, the annelids were subjected to three 2 hour changes of dioxane and three 20 minute changes in paraffin. The organisms were next imbedded in paraffin and sectioned. A series of frontal, longitudinal and transverse sections were prepared and stained with Delafield's hematoxylin. As the results of the following procedure were quite satisfactory, no other stain was attempted.

1.	xylol	5 minutes
2.	xylol	3 minutes
3.	absolute alcohol	5 minutes
4.	95% alcohol	5 minutes
5.	85% alcohol	3 minutes
6.	70% alcohol	3 minutes
7.	50% alcohol	3 minutes
8.	distilled water	3 minutes
9.	hematoxylin (Delafields)	4 minutes
10.	running water rinse	5 minutes
11.	50% alcohol	3 minutes
12.	70% alcohol	3 minutes
13.	eosin (y)	1½ minutes
14.	80% alcohol	3 minutes
15.	95% alcohol	5 minutes
16.	100% alcohol	5 minutes
17.	xylol	3 minutes
18.	xylol	5 minutes
19.	mount in permount	

The stock hematoxylin stain was diluted 1:4 for use in this technique.

APPENDIX II

A. MEASUREMENTS OF CRYPTOBIA BULLOCKI

Air Dried, Methyl Alcohol, Giemsa Stain

Slide No.	Body Length	Body Width	Ant. Flag.	Post. free Flag.	Length Kineto.	Width Kineto.	Length Nucleus	Width Nucleus	Distance from Anterior End Nucleus	Distance from Anterior End Kineto.
A163	14.1	1.8	10.3	9.7	1.9	1.2	3.0	1.7	3.4	1.2
A162	16.4	3.4	11.7	7.4	2.9	1.0	3.0	1.7	5.0	1.4
A165	13.0	2.5	10.5	11.0	2.6	1.1	2.5	0.6	3.3	1.0
A165	20.2	2.8	14.3	12.0	3.1	0.9	3.0	1.3	4.5	1.6
A166	19.0	4.5	15.6	14.0	4.1	1.8	4.7	3.8	5.5	1.9
A166	13.7	1.9	10.0	7.0	3.5	1.5	3.0	1.2	4.8	2.5
A145	15.6	2.6	10.2	9.1	2.8	0.7	3.1	1.3	4.0	1.0
A166	16.2	3.3	15.0	9.8	3.5	2.0	3.0	1.8	4.4	1.4
A166	17.8	1.4	10.0	8.9	2.8	1.2	2.7	1.4	4.7	1.8
A245	18.0	2.9	19.2	9.5	4.1	1.3	4.2	2.0	5.3	1.9
A161	19.7	2.2	13.2	8.0	3.8	1.1	3.0	1.4	4.6	1.4
A161	15.4	1.8	13.3	6.8	3.5	0.9	3.9	1.0	4.2	1.3
A161	18.5	2.5	12.8	7.0	2.3	1.3	3.7	1.4	6.0	2.6
A153	19.5	1.2	8.3	4.4	2.8	0.8	3.4	0.8	4.4	1.0
A161	17.5	1.8	12.3	6.9	3.3	1.5	3.8	1.1	6.3	2.7
A166	20.2	2.2	12.2	8.7	4.3	0.9	4.1	1.3	5.3	1.0
A300	16.1	3.0	13.7	8.1	2.8	0.8	2.7	1.3	2.7	2.4
A300	20.5	2.8	16.2	9.8	3.1	1.1	6.1	1.8	3.3	1.1
E124	21.5	2.4	16.2	8.8	3.5	1.3	4.3	1.5	0.9	4.6
E124	19.5	2.8	14.2	6.1	4.0	0.8	3.7	1.5	3.8	1.5
A324	17.3	3.0	10.6	9.3	3.8	1.0	3.5	1.7	3.9	1.4
A322	19.9	1.8	14.7	6.3	2.8	0.8	2.9	1.0	4.5	1.8
A324	20.5	3.4	16.1	8.6	4.0	1.7	5.1	1.8	4.8	2.4
A324	16.8	2.4	13.6	7.3	2.9	0.9	2.1	1.2	4.2	1.5

A. MEASUREMENTS OF CRYPTOBIA BULLOCKI continued

Slide No.	Body Length	Body Width	Ant. Flag.	Post. Free Flag.	Length Kineto.	Width Kineto.	Length Nucleus	Width Nucleus	Distance from Anterior End Nucleus	Distance from Anterior End Kineto.
A322	17.7	1.8	12.6	6.1	2.5	0.9	3.4	0.8	3.4	0.8
A320	16.8	2.5	13.0	10.4	2.9	1.3	2.8	1.8	5.7	1.6
A320	17.2	2.7	13.1	7.9	3.2	0.8	4.0	1.3	3.8	1.2
A320	15.8	2.8	12.0	8.4	2.6	0.8	3.2	1.9	4.2	1.4
A320	15.4	3.0	8.5	8.2	2.7	0.8	2.8	1.5	4.4	1.2
A320	16.0	2.8	12.4	9.1	3.0	0.8	3.7	1.4	4.4	0.9
A320	16.6	3.1	13.3	9.5	2.7	0.9	3.8	1.4	4.9	1.5
A320	18.8	3.2	11.7	7.3	3.4	0.8	3.5	1.9	4.5	0.9
A327	14.9	3.2	11.6	8.4	2.0	0.6	3.1	1.2	3.2	1.8
A327	16.5	3.7	12.8	10.7	2.3	1.1	2.9	1.7	4.4	1.6
A327	18.9	3.4	15.1	9.6	2.8	1.1	3.0	1.2	4.1	0.5
A327	18.5	2.3	14.0	8.8	3.5	0.6	3.5	1.4	3.8	0.9
A307	14.0	3.3	13.1	12.6	2.7	1.6	3.4	1.5	7.9	1.8
A240	21.1	2.0	13.1	7.1	3.8	1.2	4.5	1.4	3.9	1.3
A318	19.7	2.8	14.2	6.0	3.3	0.7	4.0	1.3	3.8	1.7
A318	14.0	2.5	11.9	7.8	5.1	0.8	3.2	1.3	4.1	1.2
A318	21.0	3.4	11.8	9.2	4.3	0.9	3.2	1.7	4.9	1.8
A318	14.3	2.2	11.1	6.8	5.2	1.0	3.8	1.3	4.0	1.8
A318	18.5	3.8	12.8	8.8	4.3	1.2	3.2	2.0	4.2	2.1
A332	14.4	2.8	8.7	6.2	2.3	0.9	2.5	1.4	4.0	1.2
G101	19.4	1.8	7.8	8.5	4.1	0.9	2.1	1.2	5.1	1.2
G101	19.2	2.7	15.1	10.5	4.6	0.9	3.8	1.6	5.0	1.1
A307	19.7	3.0	12.9	7.2	3.8	1.6	4.3	1.2	4.5	2.0
A307	17.4	2.9	11.9	6.1	3.4	0.9	4.0	1.2	4.4	1.3
A307	19.9	3.3	14.1	6.7	3.4	1.4	4.1	1.2	4.3	3.0
A327	18.4	3.6	15.4	6.5	3.8	1.2	3.2	1.3	3.7	1.0
A327	15.1	3.5	15.3	15.7	2.2	1.0	3.0	1.5	4.1	1.2

A. MEASUREMENTS OF CRYPTOBIA BULLOCKI continued

Slide No.	Body Length	Body Width	Ant. Flag.	Post. Free Flag.	Length Kineto.	Width Kineto.	Length Nucleus	Width Nucleus	Distance from Anterior End Nucleus	Distance from Anterior End Kineto.
A327	19.6	3.3	12.8	7.9	2.8	1.2	3.4	1.1	4.5	1.7
A332	14.5	3.0	9.9	7.8	2.0	1.0	2.4	1.4	4.4	1.7
A332	17.0	3.2	14.6	10.2	3.4	0.9	3.0	1.8	3.9	1.7
A332	13.4	2.3	13.3	7.2	1.8	0.9	2.1	2.0	4.6	1.0
A332	17.8	3.9	13.0	7.3	3.0	1.2	3.1	1.8	3.3	0.9
A327	16.7	2.7	11.0	8.4	3.3	0.8	3.5	1.0	4.5	1.4
A327	16.1	2.6	11.7	5.6	2.8	1.3	2.2	1.9	4.2	0.6
G101	20.2	2.5	11.3	6.1	4.0	0.8	5.9	1.2	5.7	1.3
G101	21.4	3.1	14.6	7.2	4.5	1.0	6.3	1.2	4.0	1.4
G101	21.0	2.4	11.9	6.0	4.3	1.2	6.8	0.8	4.8	1.2
G101	19.8	2.5	13.8	6.9	3.2	0.8	3.7	1.1	4.8	1.7
A333	20.4	2.4	15.4	7.4	3.1	1.0	3.3	1.4	5.4	2.1
A316	13.7	2.5	18.5	10.0	1.7	1.1	1.8	1.7	2.3	0.8
A305	17.9	2.6	13.3	9.2	4.1	1.3	4.0	1.0	3.9	1.0
A305	18.2	2.1	11.8	5.6	4.0	1.3	3.8	1.2	4.6	1.2
A305	17.3	2.1	11.5	5.0	2.1	1.0	2.2	1.1	4.8	1.4
A305	16.5	2.8	12.0	6.5	2.2	1.8	2.1	1.3	3.8	0.7
A316	15.8	2.0	12.7	7.3	3.5	1.6	4.1	0.9	2.8	0.8
A316	15.2	1.5	11.5	6.8	1.9	1.5	3.0	1.3	4.5	0.8
A321	14.8	4.1	10.0	6.8	2.5	1.0	2.5	1.8	4.5	1.6
A321	17.7	2.4	13.0	5.4	2.5	0.9	2.2	1.7	4.8	1.0
A321	17.9	3.0	13.8	8.9	3.0	1.0	2.4	1.7	5.4	1.9
A321	19.3	3.5	12.6	10.7	3.7	1.2	3.2	1.7	4.4	1.4
A321	19.8	2.4	12.3	9.0	3.5	0.9	3.8	1.3	3.5	1.7
A333	22.2	2.9	14.7	5.4	4.2	1.0	5.2	1.6	5.6	2.3
A333	15.9	2.8	12.9	6.2	3.5	1.0	2.2	1.6	3.9	1.0
A321	19.1	2.5	13.1	8.3	3.0	1.5	2.9	1.7	5.1	1.3

A. MEASUREMENTS OF CRYPTOBIA BULLOCKI continued

Slide No.	Body Length	Body Width	Ant. Flag.	Post. Free Flag.	Length Kineto.	Width Kineto.	Length Nucleus	Width Nucleus	Distance from Anterior End Nucleus	Distance from Anterior End Kineto.
A334	17.5	3.8	12.2	9.4	4.9	1.1	4.9	1.5	2.5	2.6
A149	18.0	1.9	14.8	8.0	3.8	1.0	2.8	0.9	3.9	1.3
A149	17.3	2.1	16.6	11.5	4.8	1.0	3.3	1.1	4.9	1.8
A149	20.8	1.9	15.0	10.5	4.4	1.2	4.4	1.4	4.0	0.9
A266	18.6	3.3	16.9	8.8	3.7	1.3	2.9	2.1	5.1	1.6
A153	16.2	3.5	11.1	9.6	2.8	1.0	3.2	1.9	3.5	1.4
A149	18.2	3.1	15.5	8.8	4.9	1.1	3.9	1.4	2.2	2.0
A145	13.8	1.8	11.1	8.7	3.1	0.8	2.7	1.1	2.8	2.1
A166	20.4	2.2	19.1	11.6	5.5	1.8	4.1	1.5	6.3	1.7
A108	22.6	2.7	10.0	7.4	3.9	0.8	4.4	1.3	5.1	1.8
A108	23.1	2.4	11.9	7.2	5.0	1.5	5.4	0.8	4.1	1.3
A151	18.2	2.0	12.8	9.5	3.6	1.2	4.0	1.4	4.7	1.4
A153	17.6	1.8	11.0	8.3	3.8	1.1	3.3	1.1	3.4	1.4
A326	18.2	2.7	11.9	5.7	3.1	1.0	4.8	1.1	3.8	1.3
A334	19.6	2.8	11.9	9.4	3.8	0.9	4.1	1.6	5.7	2.6
A326	16.9	3.0	11.9	7.7	3.5	0.8	3.0	1.8	3.8	0.9
A326	12.5	3.2	10.8	7.3	3.0	0.8	3.1	1.2	4.0	1.5
A326	13.3	3.0	13.2	8.0	2.1	1.3	3.3	1.2	2.9	1.8
A326	14.2	3.9	16.9	10.1	3.3	1.5	3.8	1.7	4.0	1.7
A337	16.5	2.5	13.4	11.0	3.6	0.8	3.6	1.3	4.2	1.3
A337	20.0	2.1	14.8	11.3	3.3	0.9	2.4	1.5	2.9	0.9
A337	17.8	1.8	13.8	9.5	2.4	1.1	2.2	1.3	3.7	1.3
A337	17.8	1.8	13.8	5.1	2.8	1.0	2.2	1.3	3.9	0.8
A337	19.8	2.7	13.0	7.3	2.3	0.9	2.0	2.0	3.7	1.5
A330	15.7	2.2	15.9	9.1	3.0	1.3	3.3	1.0	3.0	0.1

B. MEASUREMENTS OF CRYPTOBIA BULLOCKI

Osmic Fumes, Absolute Alcohol, Giemsa Stain

Slide No.	Body Length	Body Width	Ant. Flag.	Post. Free Flag.	Length Kineto.	Width Kineto.	Length Nucleus	Width Nucleus	Distance from Anterior End Nucleus	Distance from Anterior End Kineto.
A346	11.6	3.3	7.1	7.2	1.3	1.2	1.9	1.7	4.6	1.2
A347	19.2	4.1	12.6	9.7	3.3	1.8	4.0	1.8	4.0	2.0
A339	18.0	2.9	10.8	11.2	1.6	1.0	3.0	1.2	5.0	0.5
A339	12.3	4.0	13.0	8.2	2.2	1.3	2.2	1.5	3.4	2.0
A346	14.2	3.7	12.3	7.6	2.8	1.1	2.5	1.0	4.8	1.0
A274	14.5	3.0	11.5	7.5	2.0	1.5	3.0	1.0	3.5	1.0
A1	18.2	4.6	13.2	8.8	3.8	1.5	4.9	1.8	4.7	1.8
A273	16.2	3.3	11.5	6.8	3.0	1.7	3.1	1.6	5.0	1.2
A1	14.9	4.0	13.6	7.2	3.8	1.6	2.6	2.2	4.4	0.8
A1	17.6	5.4	15.2	8.2	3.4	1.2	3.3	1.3	4.5	1.2
A1	17.6	4.8	16.2	8.2	3.6	1.4	3.1	1.8	5.0	1.5
A1	18.6	4.8	16.2	8.3	4.0	1.6	3.3	1.6	5.1	2.0
A1	15.9	4.9	16.0	9.1	3.8	1.9	4.3	1.2	4.8	1.5
A1	17.4	4.0	16.5	8.9	5.0	1.4	3.8	1.0	4.1	1.3
A1	16.2	6.0	5.8	8.9	2.2	3.6	2.8	1.6	4.2	1.8
A1.	19.2	5.0	13.8	18.2	4.5	1.3	3.0	1.8	4.0	1.0
A345	20.0	4.0	10.3	7.0	4.8	1.5	2.7	2.6	5.0	2.0
A342	18.4	4.1	14.0	8.0	2.8	1.5	4.8	1.7	4.3	1.8
A342	14.9	3.0	11.6	8.8	2.0	1.7	3.0	1.8	4.8	1.0
A342	21.7	3.5	12.9	4.6	5.3	1.4	4.2	1.8	4.7	1.8
A338	15.1	4.4	15.0	9.9	5.7	3.1	5.2	1.6	4.3	1.0
A342	13.3	4.9	14.3	7.7	4.3	1.6	3.6	2.3	4.3	0.9
A342	15.8	4.9	13.4	7.4	4.3	1.8	4.0	1.4	4.6	1.2
A342	16.6	4.1	12.0	9.0	4.8	1.1	3.2	2.1	3.8	1.6
A342	14.4	4.6	13.7	10.0	3.8	1.9	3.7	1.6	3.2	0.2
A342	13.1	4.9	13.8	9.5	2.0	1.6	3.9	2.1	4.6	0.1

B. MEASUREMENTS OF CRYPTOBIA BULLOCKI continued

Slide No.	Body Length	Body Width	Ant. Flag.	Post. Free Flag.	Length Kineto.	Width Kineto.	Length Nucleus	Width Nucleus	Distance from Anterior End Nucleus	Distance from Anterior End Kineto.
A338	19.1	4.0	16.1	9.8	5.1	1.4	3.9	1.6	4.0	1.3
L7	15.0	3.4	13.0	7.8	5.1	1.8	5.4	1.6	6.2	1.1
L7	14.2	3.0	9.0	9.8	3.1	1.3	3.0	1.8	3.5	0.9
L7	18.0	5.1	9.3	10.9	4.0	1.5	4.2	2.3	3.0	2.2
L7	17.4	5.3	12.8	8.6	4.6	1.9	4.7	2.0	2.2	0.1
L7	17.4	5.3	12.8	8.6	4.6	1.9	4.7	2.0	2.2	0.1
L7	15.7	6.0	15.0	6.5	3.8	2.0	3.2	2.0	2.3	2.1
A347	16.4	5.0	14.3	10.0	4.3	1.3	4.0	2.1	4.5	1.0
A347	14.0	4.0	12.4	8.0	4.1	1.5	3.7	2.4	4.5	1.5
A347	10.9	3.7	8.5	8.2	2.9	1.5	2.7	2.0	3.0	0.2
A339	15.8	3.0	13.0	8.8	2.4	1.1	2.3	1.7	5.2	1.8
A347	15.0	4.4	12.3	5.1	3.3	1.4	3.5	1.6	1.5	0.7
A347	15.7	3.6	14.3	8.4	4.0	1.8	3.4	2.5	5.4	1.7
A339	13.2	3.1	10.6	8.6	1.9	1.2	2.3	2.2	2.0	1.5
A339	15.5	3.8	9.8	7.8	3.4	0.8	3.2	1.7	1.2	1.1
A344	19.5	5.5	16.3	8.8	5.5	2.5	4.0	2.4	4.6	2.0
A344	20.9	5.5	17.5	10.4	2.8	2.0	3.9	1.6	5.3	3.0
A344	19.6	4.3	15.6	10.0	5.7	2.0	5.6	2.5	2.8	2.3
A344	20.4	5.3	16.2	9.5	4.4	1.8	4.3	2.5	5.9	2.5
A344	21.0	5.0	15.8	8.4	4.8	2.7	4.3	2.3	4.8	0.6

APPENDIX III

PLATES AND FIGURES

Plate I. Giemsa Stain

Figure 1. Small size organism
2. Medium size organism
3. Large size organism
4. Narrow form
5. Short, stubby form
6. From a leech
7. From a leech
8. Erythrocyte

Plate II. Taliaferro's Stain

Figure 1. Large size organism
2. Medium size organism
3. Small size organism
4. Ameboid form
5. Ameboid
6. From brain of flounder
7. Erythrocyte

Plate III.

Figure 1. Undulating membrane - Giemsa
2. Undulating membrane - Giemsa
3. Blepharoplast - Iron Alum Hematoxylin
4. Blepharoplast - Iron Alum Hematoxylin

5. Blepharoplast - Iron Alum Hematoxylin

6. Type specimen - Giemsa stain

7. Erythrocyte

Plate IV.

Figure 1. The invertebrate host of Cryptobia bullocki

Plate V.

Figure 1. Geographic distribution of Cryptobia bullocki.

PLATE I

AIR DRIED, METHYL ALCOHOL FIXED, GIEMSA STAINED

Figure 1. Small size *Cryptobia bullocki*. (13.4 u by 2.3 u) From blood of a flounder

Figure 2. Medium size *Cryptobia bullocki*. (17.4 u by 2.9 u) From blood of a flounder

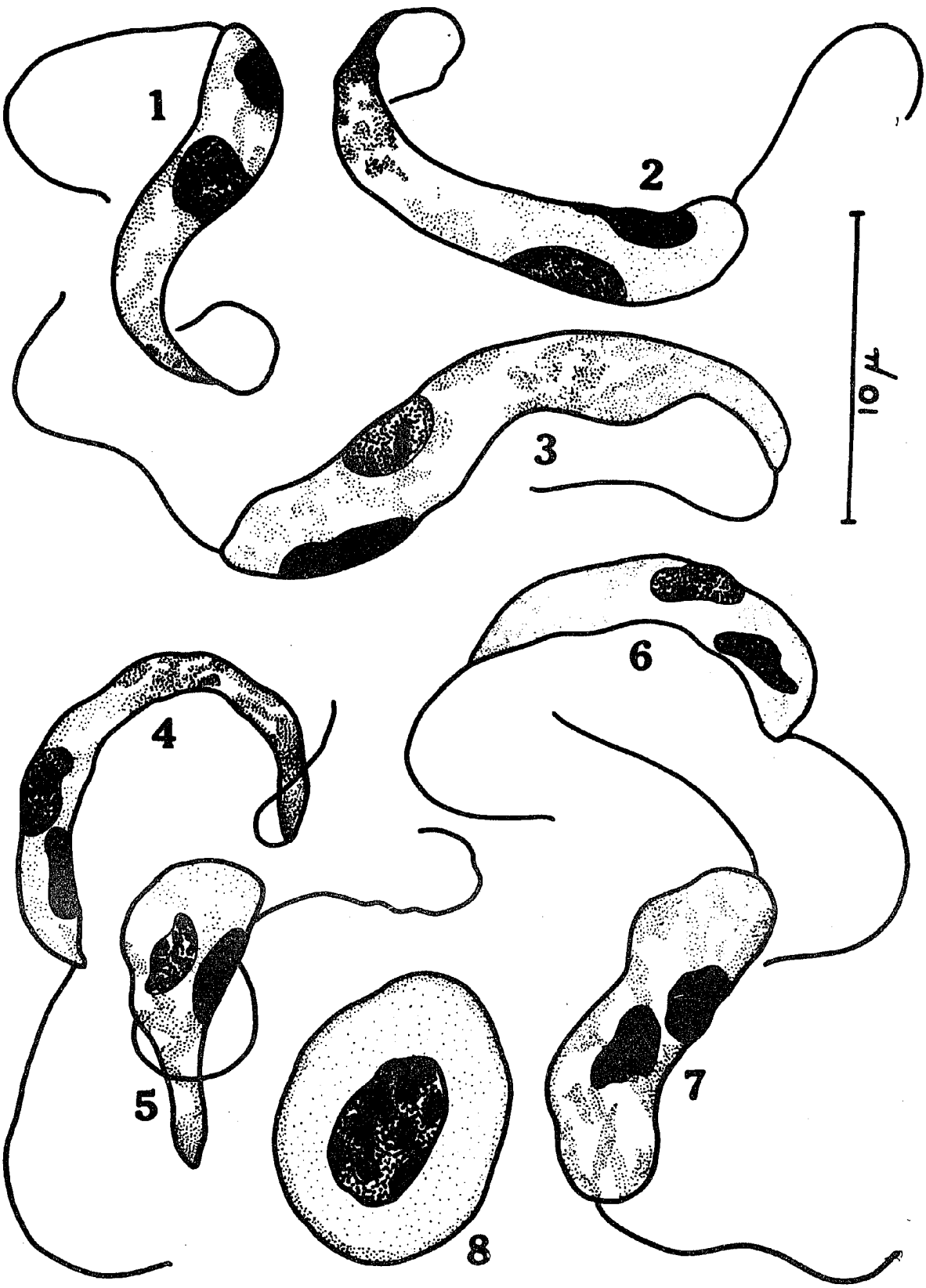
Figure 3. Large size *Cryptobia bullocki*. (21.0 u by 3.4 u) From blood of a flounder

Figure 4. Long, narrow form of *Cryptobia bullocki*. From blood of a flounder

Figure 5. Short, stubby form of *Cryptobia bullocki*. From blood of a flounder

Figures 6 and 7. *Cryptobia bullocki* from the smear of a leech. These organisms are morphologically similar to the flounder blood forms.

Figure 8. Erythrocytes from flounder blood.



Cryptobia bullocki n. sp.

PLATE 2

OSMIC FUMES, ABSOLUTE ALCOHOL FIXED, GIEMSA STAIN

(Taliaferro's method)

Figure 1. Large size Cryptobia bullocki. (21.0 u by 5.0 u) From blood of a flounder.

2. Medium size Cryptobia bullocki. (15.9 u by 4.0 u) From blood of a flounder.

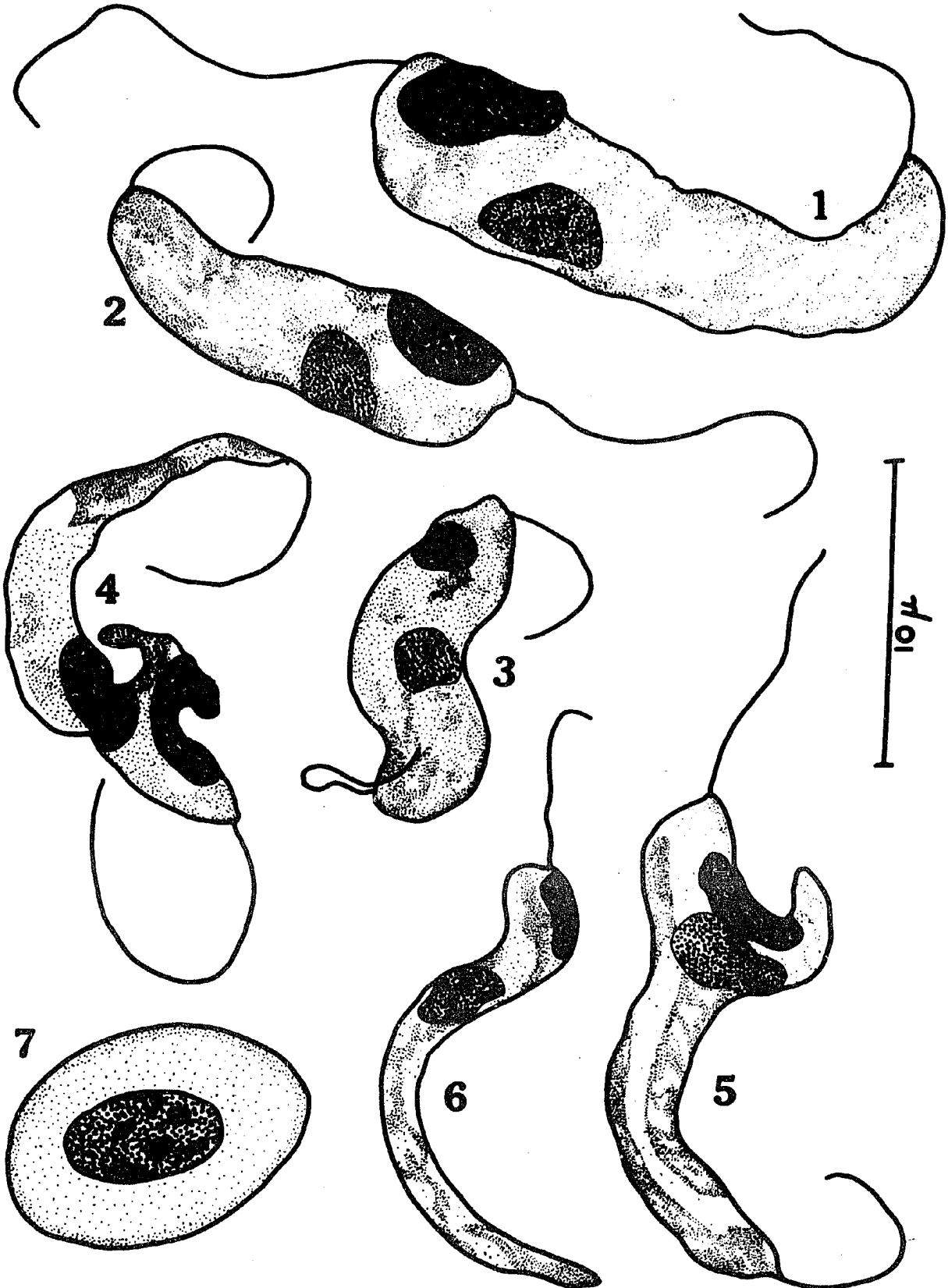
3. Small size Cryptobia bullocki. (11.6 u by 3.3 u) From blood of a flounder.

4. Ameboid form of Cryptobia bullocki. This organism may possibly be dividing. From blood of a flounder.

5. Ameboid form of Cryptobia bullocki. These forms, frequently seen in fresh smears, may be mistaken for dividing organisms.

6. Cryptobia bullocki from the brain of a flounder. The organism depicted appears to have only one flagellum, though two flagella are usually present on the parasites found in the brain.

7. Erythrocyte from flounder blood.



Cryptobia bullocki n. sp.

PLATE 3

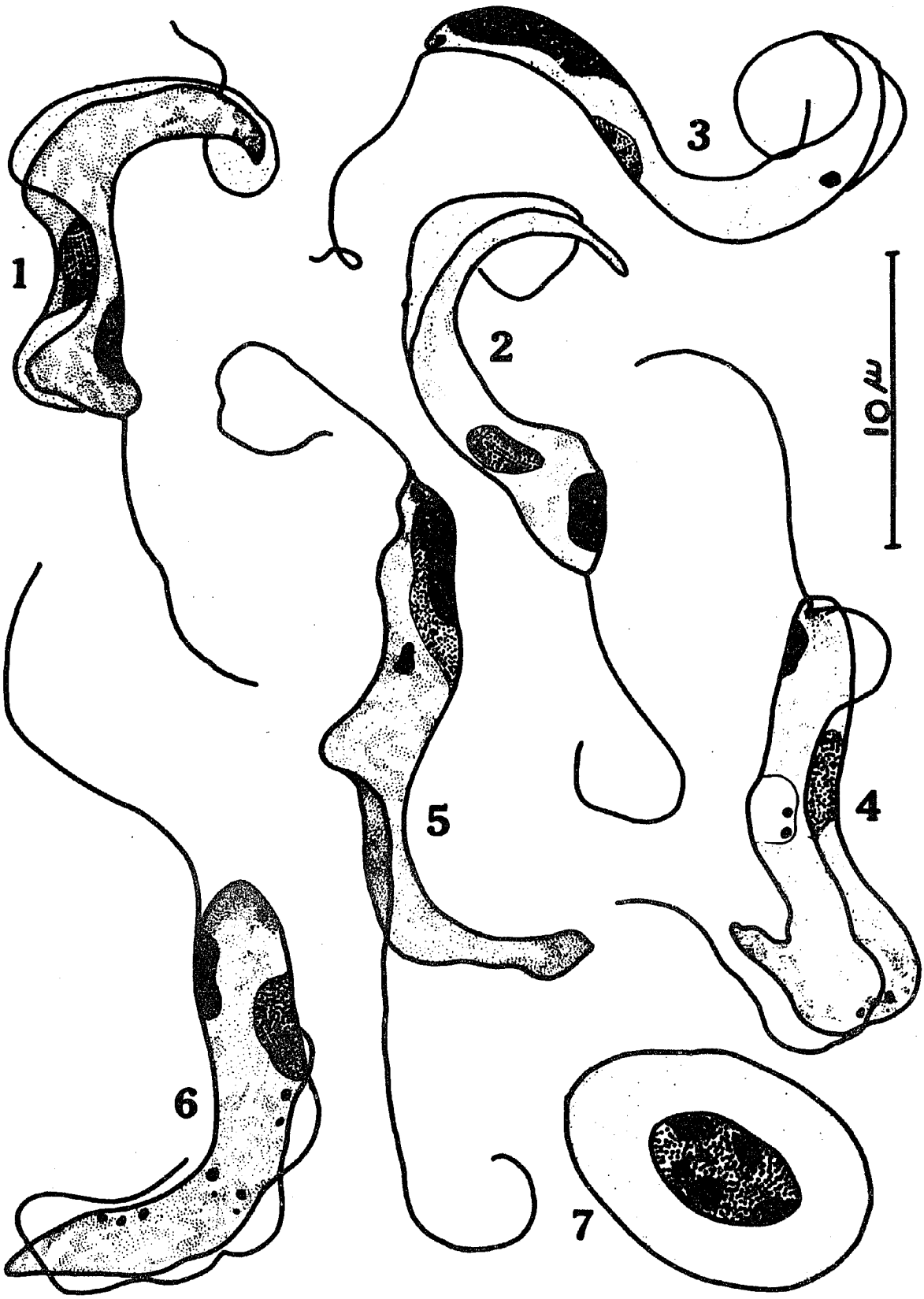
Figure 1. *Cryptobia bullocki*, Giemsa stain. The undulating membrane bordered by the posterior flagellum is clearly seen.

Figure 2. *Cryptobia bullocki*, Giemsa stain. The undulating membrane is visible on only the posterior portion of the parasite.

Figures 3, 4, and 5. *Cryptobia bullocki*, Iron Alum Hematoxylin. The blepharoplast, anterior to the kinetoplast, is demonstrated with iron alum hematoxylin stain, but the nucleus of the organism is often not apparent.

Figure 6. *Cryptobia bullocki*, Giemsa stain. Type specimen.

Figure 7. Erythrocyte from the blood of the flounder.



Cryptobia bullocki n. sp.

PLATE 4

Figure 1. The invertebrate host of *Cryptobia bullocki*. This leech is provisionally designated as *Piscicola rapax* Verrill, 1873 (Moore, 1898). This figure is drawn from a cleared whole mount of the specimen.

PLATE 4

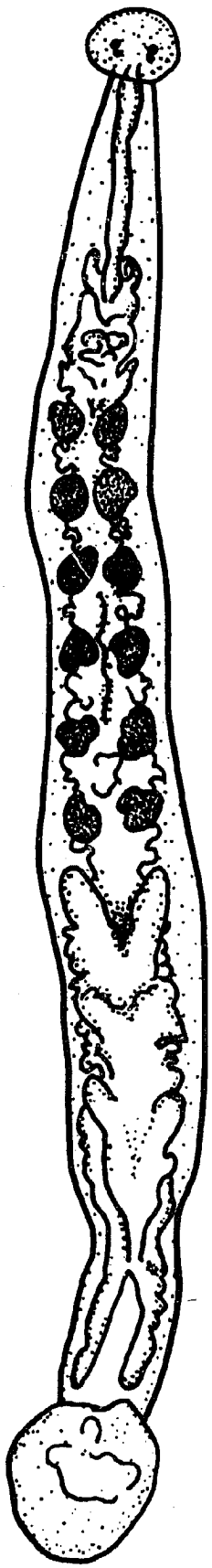


Plate 5

Figure 1. Locations for the collection of Cryptobia bullocki. The flagellate was found in the blood of marine fishes from Great Bay, New Hampshire; Portsmouth Harbor, N. H.; Hampton Harbor, N. H.; Newburyport, Mass.; York, Maine and Kennebunkport, Maine.

PLATE 5